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GENETIC AND PHYSIOLOGIC STUDIES OF *BACILLUS ANTHRACIS* RELATED TO DEVELOPMENT OF AN IMPROVED VACCINE

ANNUAL AND FINAL REPORT

CURTIS B. THORNE

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This is the fifth and last Annual Progress Report of research carried out under the above referenced contract. The primary objective of the research is to gain information and develop genetic systems that will contribute to development of an improved vaccine for anthrax. During the year represented by this report our research concentrated largely on (i) physical and genetic analysis of the Bacillus anthracis toxin plasmid, pXO1, based largely on results of studies with mutants produced by transposon mutagenesis; (ii) transposon mutagenesis of the B. anthracis capsule plasmid, pXO2, and characterization of the various mutants obtained; and (iii) further characterization of the conjugative plasmid, pLS20, of Bacillus subtilis (natto) and its ability to transfer plasmids among strains of B. anthracis, B. cereus, B. subtilis, and B. thuringiensis.

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During the past year more mutants were isolated which exhibited alterations in phenotypic characteristics associated with the presence of pXO1.1. (According to our terminology pXO1 designates the toxin plasmid found in the wild-type Weybridge strain and pXO1.1 designates the toxin plasmid found in Weybridge A mutants. Although the two plasmids are very similar, pXO1.1 carries one or more mutations which confer upon the host strain phenotypes that are somewhat different from those exhibited by strains carrying pXO1). Several more strains carrying pXO1::Tn*917* derivatives were isolated which showed alterations in toxin production, sensitivity to bacteriophage, and extent of sporulation. One very interesting transposant, Weybridge A UM23 tp62, has the phenotype PA^{a+} (ability to produce protective antigen in the absence of added bicarbonate or CO_2). It produces more protective antigen in both the presence and absence of bicarbonate and CO_2 than the parent Weybridge A strain. It is conceivable that this mutant would be useful in production of the protective antigen vaccine preparation that is currently available in the United States. Two other transposants, Weybridge A UM23 tp49 and tp71, are also very interesting. They have DNA deletions within pXO1.1 of greater than 100 kb, and they have the phenotypes PA^{+} and PA^{-}, respectively. Results of DNA-DNA hybridizations showed that the plasmid from tp49 contained the structural genes for PA and LF (lethal factor) and the structural gene for EF (edema factor) was missing. In the same tests, the plasmid from tp71 was found to contain the structural gene for LF; the structural genes for PA and EF were missing. These mutants may be useful to those investigators who are interested in study LF and those who wish to obtain protective antigen preparations free of edema factor. Hopefully, studies currently under way will confirm that the antigens produced in broth culture by the two transposants are consistent with the results of the hybridization studies.

B. anthracis plasmid pXO2 carries information for synthesis of the D-glutamyl polypeptide capsule, one of the organism's virulence factors. Previous work in our laboratory demonstrated that strains cured of this plasmid were noncapsulated and capsule synthesis was restored upon introduction of pXO2 into cured cells. We have used the transposition selection vector pTV1 to generate pXO2::Tn*917* derivatives which may have altered phenotypes with respect to capsule formation. These transposants are being analyzed for any alteration of capsule formation or other phenotypes that may be associated with pXO2. The various phenotypes of the insertion mutants thus far found can be described as follows: (1) Noncapsulated mutants (Cap^{-}) which still retain pXO2 but produce noncapsulated colonies under all growth conditions. (2) Strains that produce capsules only when grown in media containing bicarbonate and incubated in a CO_2-rich atmosphere (Cap^{c+}). This is the phenotype of cells carrying wild-type pXO2. However, a few of these insertion mutants have the very interesting characteristic of not being able to grow in the absence of CO_2. Also some of these Cap^{c+} transposants appear to be polypeptide overproducers and resemble *Bacillus licheniformis* with respect to colony morphology. (3) Mutants that produce capsules when grown in air in the absence of bicarbonate (Cap^{a+}). A few of these have the unusual characteristic of being unable to grow in a CO_2-rich atmosphere.

A small amount of effort was spent on further characterization of the *Bacillus subtilis* (natto) fertility plasmid, pLS20, which encodes functions required for conjugal transmission of plasmid DNA among a variety of *Bacillus* species, including *B. anthracis*. Cells of *B. anthracis* that contained pLS20 appeared more dense by electron microscopy than cells not carrying the plasmid, suggesting that they may have a surface component not present in wild-type cells. Cells of normally motile species appeared to be unable to synthesize flagella when they were infected with pLS20. A physical and functional map of pLS20 was constructed based on results obtained with Tn*917* insertion mutants and some deletion mutants derived from them.

This document also includes a Final Report which is a narrative summary of the research carried out during the five-year period of the contract. It is accompanied by pertinent references to specific annual progress reports and other publications.

SUMMARY

Physical analysis of the Bacillus anthracis toxin plasmid, pXO1.1, has confirmed the regions of pXO1.1 involved in toxin production and further defined the regions surrounding the toxin genes. DNA-DNA hybridization analysis confirmed that the restriction fragments of pXO1.1 which contain the toxin structural genes corresponded to similar fragments on the restriction map of pXO1 prepared by D. Robertson. DNA-DNA hybridization analysis of the Tn917-tagged deletion derivative from Weybridge A UM23 tp21, which has the interesting phenotype PA^- LF^+ EF^+, showed that the 34.8-kb, 13.9-kb, and 6.0-kb BamHI fragments, which are adjacent to or carry the toxin structural genes, have incurred deletions which joined the 34.8-kb and the 13.9-kb fragments together to form a new 44.2-kb BamHI fragment. The results obtained from analysis of the plasmid from UM23 tp21 are consistent with the PA^- LF^+ EF^+ phenotype observed in this mutant.

During the past year more mutants were isolated which exhibited alterations in phenotypic characteristics associated with the presence of pXO1.1. Several more strains carrying pXO1.1::Tn917 were isolated which showed alterations in toxin production, sensitivity to bacteriophage, and extent of sporulation. One transposant of interest, UM23 tp62, which has the phenotype PA^{a+} (ability to produce PA in the absence of added bicarbonate or CO_2), produced more PA and LF in both the presence and absence of added bicarbonate than the parent strain, UM23. UM23 tp62 also appeared to sporulate less frequently than UM23. The plasmid from tp62 was transferred to B. anthracis strains cured of pXO1.1 or pXO1 by conjugation. Phenotypes similar to those observed in tp62 were also observed in these transcipient strains indicating that the altered phenotypes are indeed caused by Tn917 insertion into pXO1.1. Restriction analysis of the plasmid from tp62 showed that the 34.8-kb BamHI fragment and the 9.3-kb PstI fragment which is nested within the 34.8-kb BamHI fragment are altered. These results suggested that this region may be involved in the regulation of toxin production and may also be involved in the regulation of sporulation. From analysis of transposants which failed to produce the toxin components, we previously showed that the 13.9-kb BamHI may also be involved in the regulation of toxin production.

Two of the transposants, UM23 tp49 and tp71, have deletions within pXO1.1 of greater than 100 kb, and they have the phenotypes PA^+ and PA^-,

respectively. As determined from summation of restriction fragments, the deletion in the plasmid from tp49 included approximately 49 kilobases and that from tp71 included about 47.5 kilobases of DNA. DNA-DNA hybridizations confirmed that the plasmid from tp49 contained the structural genes for PA and LF, while the plasmid from tp71 contained only the structural gene for LF. UM23 tp49 and tp71 also showed alterations in sensitivity to bacteriophage and in extent of sporulation.

Another B. anthracis mutant, UM23C1 tds1, containing a Tn917-tagged deletion derivative (81.1 kb) obtained previously by CP-51-mediated transductional shortening, failed to produce the toxin components; however, the extent of sporulation and sensitivity to CP-51 were similar to the phenotypes observed with the pXO1.1-containing strain, UM23. UM23C1 tds1 cured of the deletion derivative exhibited phenotypes similar to those of the pXO1.1-cured strain, UM23C1. The 81.1-kb plasmid from tds1 was transferred to B. anthracis UM44-1C9 by CP-51 mediated transduction. The resulting transductants exhibited characteristics very similar to those of tds1. These results show that the phenotypes observed in tds1 are plasmid-derived and that the 81.1-kb plasmid from tds1 contains the regions involved in sporulation and sensitivity to bacteriophage.

The transposition selection vector, pLTV3, was introduced into B. anthracis UM23-1. The purpose of introducing pLTV3 into pXO1.1-containing strains was to generate Tn917-LTV3-tagged pXO1.1 derivatives by transposon mutagenesis, not only to increase our library of insertional mutants, but also to create in vivo gene fusions, taking advantage of the fact that Tn917-LTV3 contains a promoterless lacZ gene. Thus, we hope to isolate mutants containing insertions in cryptic genes.

B. anthracis plasmid pXO2 carries information for synthesis of the D-glutamyl polypeptide capsule, one of the organism's virulence factors. Previous work in our laboratory demonstrated that strains cured of this plasmid were noncapsulated, and capsule synthesis was restored upon introduction of pXO2.

We have used the transpsosition selection vector, pTV1, to generate pXO2::Tn917 derivatives which have altered phenotypes with respect to capsule formation. Approximately 65% of the transposants thus far examined carried Tn917 in pXO2 as confirmed by DNA-DNA hybridization experiments using ^{32}P labelled Tn917 as the probe. These transposants are being analyzed for any

alteration of capsule formation or other phenotypes associated with pXO2. The various phenotypes of the insertion mutants with respect to capsule synthesis can be described as follows: (1) Noncapsulated strains (Cap^-) which still retain pXO2 but produce rough colonies under all growth conditions. (2) Strains that produce capsule only when grown in media containing bicarbonate and incubated in a CO_2-rich atmosphere (Cap^{c+}). This is the phenotype of cells carrying wild-type pXO2. However, a few of these mutants have the very interesting characteristic of not being able to grow in the absence of CO_2. Also some of these Cap^{c+} mutants appear to be polypeptide overproducers and resemble Bacillus licheniformis with respect to colony morphology. (3) Strains that produce capsules when grown in air in the absence of bicarbonate (Cap^{a+}). A few of these have the unusual characteristic of being unable to grow in a CO_2-rich atmosphere.

Evidence that the mutant phenotypes were the result of Tn917 insertions and not spontaneous mutations was obtained by transduction and conjugation. Tn917-tagged pXO2 derivatives from transposants were transferred by CP-51 mediated transduction or by the B. thuringensis conjugation system to strains previously cured of pXO2. All transcipients that inherited the pXO2::Tn917 derivatives exhibited the donor phenotype.

The 64.2-kb Bacillus subtilis (natto) plasmid pLS20 encodes functions required for conjugal transmission of plasmid DNA among a variety of Bacillus species. Localization of the transfer region on this plasmid was accomplished via the analysis of insertion and deletion mutants. Utilization of the temperature-sensitive transposition selection vector pTV1 allowed the isolation of a collection of pLS20::Tn917 derivatives. Insertion of Tn917 outside the 10.8-kb BglII fragment of pLS20 did not affect the transfer abilities of the host cells. Insertion of the transposon within the 10.8-kb BglII fragment led to the identification of two distinct regions of pLS20 involved in plasmid-mediated DNA exchange. Insertions into one portion of the 10.8-kb BglII fragment abolished the ability of pLS20 to transfer itself but did not affect the plasmid's ability to mobilize the tetracycline resistance plasmid pBC16. Mutants with insertions in another region of this fragment were almost completely transfer-deficient.

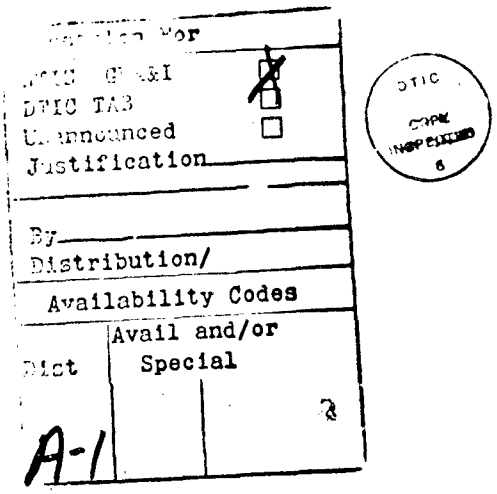
Certain of the transposon-tagged derivatives incurred specific deletions following growth of host organisms for several generations in the presence of inhibitory levels of erythromycin. Analysis of the deletion mutants revealed

that loss of ca. 16 kb of DNA encompassing the 10.8-kb BglII fragment resulted in total loss of conjugal transfer ability. Confirmation that this 10.8-kb BglII fragment contained all the DNA sequences necessary for conjugal DNA transfer came from cloning the fragment in the B. subtilis cloning vector pBD64. B. subtilis transformants carrying pBD64 containing the cloned fragment were able to transfer the recombinant plasmid by conjugation at frequencies comparable to those obtained with B. subtilis donors carrying pLS20 or nondefective pLS20::Tn917 plasmids.

The same region of pLS20 that is involved in transfer functions was also found to be involved in the suppression of motility of host organisms. Cells harboring pLS20, or the non-defective transposon-tagged derivatives, or the cloned 10.8-kb BglII fragment were non-motile on 0.4% agar plates, and electron photomicrographs revealed the absence of flagella. However, cells cured of the plasmid or cells harboring transfer-defective deletion derivatives of pLS20 were flagellated and motile. Cells of B. anthracis that contained pLS20 appeared more dense by electron microscopy than cells not carrying the plasmid, suggesting that they may have a surface component not present in wild-type cells.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.



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ANNUAL PROGRESS REPORT

This is the fifth and last annual report, together with the final report, submitted under contract DAMD17-35-C-5212. Research on the contract which began August 1, 1985 was a continuation of research previously carried out under contract DAMD17-80-C-0099.

During the year represented by this annual report our research concentrated largely on (i) physical and genetic analysis of the B. anthracis toxin plasmid, pXO1; (ii) transposon mutagenesis of the B. anthracis capsule plasmid, pXO2, and characterization of the various mutants obtained; and (iii) further characterization of the conjugative plasmid, pLS20, of Bacillus subtilis (natto) and its ability to transfer plasmids among B. subtilis, B. cereus, B. thuringiensis, and B. anthracis.

In this report our main efforts for the past year are discussed following a general description of materials and methods. Specific procedures which themselves are results of the research are described as appropriate under individual sections.

MATERIALS AND METHODS

Organisms. Table 1 lists the bacterial strains, plasmids, and bacteriophages referred to in this report.

Media. For convenience to the reader, compositions of the various culture media referred to in this report are given below. All amounts are for one liter final volume. For preparation of solid medium, 15 grams of agar (Difco) were added per liter of the corresponding broth.

NBY broth: Nutrient broth (Difco), 8 g; Yeast extract (Difco), 3 g.

NBY-MN broth: NBY broth with 25 µg of $MnSO_4.H_2O$ per ml.

Phage assay (PA) broth: Nutrient broth (Difco), 8 g; NaCl, 5 g; $MgSO_4.7H_2O$, 0.2 g; $MnSO_4.H_2O$, 0.05 g; $CaCl_2.2H_2O$, 0.15 g. The pH was adjusted to 6.0 with HCl.

Phage assay agar: For bottom agar, 15 g of agar were added per liter of phage assay broth. For soft agar, 0.6 g of agar were added per liter.

L broth: Tryptone (Difco), 10 g; Yeast extract (Difco), 5 g; NaCl, 10 g. The pH was adjusted to 7.0 with NaOH.

LPA agar: L agar containing the salts of PA broth.

LPACO_3 agar: LPA agar with 5 g of $NaHCO_3$.

LG broth: L broth with 1 g of glucose.

BHI broth: Brain heart infusion broth (Difco), 37 g.

Peptone diluent: Peptone (Difco), 10 g. Used for diluting phage and bacterial cells.

Minimal I: $(NH_4)_2SO_4$, 2 g; KH_2PO_4, 6 g; K_2HPO_4, 14 g; sodium citrate, 1 g; glucose, 5 g; L-glutamic acid, 2 g; $MgSO_4.7H_2O$, 0.2 g; $FeCl_3.6H_2O$, 0.04 g; $MnSO_4.H_2O$, 0.00025 g. The pH was adjusted to 7.0 with NaOH. The glucose and $FeCl_3$ were sterilized separately.

Minimal IC: Minimal I with 5 g of vitamin-free Casamino acids (Difco) and 10 mg of thiamine hydrochloride.

Minimal XO: To Minimal 1 were added 10 mg of thiamine hydrochloride, 200 mg of glycine, and 40 mg of L-methionine, L-serine, L-threonine, and L-proline.

CA broth is the Casamino acids medium as described by Thorne and Belton (15).

CA-agarose medium: CA-agarose medium for the detection of colonies producing protective antigen was prepared as follows: 0.75 g of agarose was added to 100 ml of CA broth (prepared as described by Thorne and Belton [15]) and the mixture was steamed until the agarose was dissolved. When the medium cooled to about 50° C, 1 ml of 20% glucose, 8 ml of 9% $NaHCO_3$, 6 ml of goat antiserum to B. anthracis, and 10 ml of horse serum were added. The medium was dispensed in petri plates (13 ml per plate) and the plates were left with their lids ajar while the agarose solidified. The plates were usable after 1 hr.

RM medium is R medium (10) as modified by Leppla (8).

Antiserum. B. anthracis antiserum was kindly supplied by personnel of USAMRIID.

Propagation of bacteriophage. Bacteriophage CP-51ts45 for transduction was propagated by picking 5 plaques from an assay plate (B. cereus 569 indicator), suspending in 5 ml of 1% peptone and filtering through a Millipore HA membrane filter. Cells were grown in 25 ml of L broth containing 0.5%

(wt/vol) glycerol at 30°C with slow shaking for 6 to 8 hours following a 10% transfer from an overnight culture in L broth with 15 µg of chloramphenicol per ml (to maintain the plasmid marker). One-half ml of cells and 0.5 ml of phage suspension were added to 3 ml of soft NBY agar and layered over freshly poured plates of NBY agar containing 0.5% glycerol. Propagation plates were incubated at 30°C for at least 30 hours and the phage from each plate was harvested in 5 ml of PA broth. Cells were removed by centrifugation at 10,000 RPM for 15 min at 15°C, and the supernatant fluid was filtered through a Millipore HA membrane filter. Dimethyl sulfoxide was added to give a final concentration of 10% and $MgSO_4$ was added to give a final concentration of 0.02 M. Lysates were tested for sterility and stored at 15°C.

Phage assays. For assaying bacteriophage CP-51ts45, 0.1 ml of B. cereus 569 spores (standard indicator) having 3 x 10^9 CFU/ml were added to 2.5 ml of soft PA agar. One-tenth ml of phage diluted in sterile 1% peptone was added to the mixture which was poured over PA agar plates. Assay plates were incubated at 30°C for 18 to 20 hours.

Transduction of pTV1 with CP-51ts45. Recipient cells for transduction of pTV1 were grown in 25 ml of BHI broth containing 0.5% glycerol (w/v) with fast shaking at 37°C for 6 to 8 hours following a 10% transfer from an overnight culture. One-tenth ml of CP-51ts45 phage lysate and 0.1 ml of recipient cells were spread together on HA Millipore filter membranes, and incubated on LE-agar (L-agar with 0.1 µg/ml of erythromycin) for 4 hours at 37°C to allow for phenotypic expression of plasmid encoded antibiotic resistance. The membranes were then transferred to L-agar plates containing selective levels of erythromycin and lincomycin (1 µg of erythromycin and 25 µg of lincomycin per ml) and incubation was continued at 37°C for 30 hours.

Test for capsule production. The ability of B. anthracis and B. cereus to produce capsules was determined by growing cells on LPA agar incubated in air for those mutants which did not require bicarbonate for capsule production, or on $LPACO_3$ agar incubated in 20% CO_2 for those strains which produced capsules only in the presence of bicarbonate. Plates were incubated at 37°C for 24 to 48 h.

Transduction of pXO2. Bacteriophage CP-51ts45 was propagated on B. anthracis and assayed on B. cereus 569. Recipient cells for transduction were grown in 250-ml flasks containing 25 ml of L broth (for B. cereus) or BHI broth with 0.5% glycerol (for B. anthracis) and incubated at 37°C on a rotary

shaker at 250 RPM. Cells from a 10% (vol/vol) transfer of a 16-h culture were grown for 5 h. Cells (0.1 ml containing approximately 10^8 CFU) and phage (0.1 ml containing approximately 5 x 10^9 PFU) were spread together on $LPACO_3$ agar. Plates were incubated at 37°C in 20% CO_2. After 3 h, 0.1 ml of phage CP-54 (3 x 10^9 PFU) was spread on the transduction plates to lyse noncapsulated cells and to allow the selection of capsulated transductants. Incubation in CO_2 was continued for 36 to 48 h.

Chromosomal transduction with CP-51ts45. Recipient cells for transduction of chromosomal markers were grown in 25 ml of L broth containing 0.5% glycerol (w/v) at 37°C with fast shaking for 6 to 8 hours following a 10% transfer from an overnight culture. One-half ml of CP-51ts45 phage lysate and 0.5 ml of recipient cells were mixed together in a 20-mm cotton-plugged tube and incubated at 37°C for 30 minutes with fast shaking. For transducing the recipient to MLS^r, 0.1 ml of the transduction mixture was spread on an HA filter membrane placed on L agar supplemented with inducing concentrations of erythromycin (0.1 ug/ml). The plate was incubated for 4 hours at 37°C to allow phenotypic expression of antibiotic resistance. The membrane was then transferred to L agar containing 1 ug of erythromycin and 25 ug of lincomycin per ml to select for Tn917. For transducing auxotrophic mutants 0.1 ml of the transduction mixture was spread directly on appropriate minimal medium to select transductants inheriting the wild-type allele. The plates were scored after 2 days of incubation at 37°C.

Detection of plasmid DNA. The procedures for extracting plasmid DNA and for electrophoresis were the same as those described in the Annual Progress Report dated July 31, 1988.

Restriction endonuclease digestions. Restriction endonuclease digestions were carried out under conditions recommended by the supplier of the enzymes. Usually 10 to 20 ul of DNA (1.0 to 1.5 ug) in TES (pH 8.0) was added to 5 to 10 units of enzyme in a 1.5 ml Eppendorf tube. Appropriate amounts of distilled water and 10X buffer were added to give a total volume of 100 ul. Reaction mixtures were incubated in a 37°C water bath for 2 to 15 h. Digests were heated at 65°C for 10 minutes to stop reactions and then resolved on agarose gels. Molecular weights of DNA fragments were determined by comparing their mobilities to those of a kilobase ladder consisting of fragments ranging in size from 0.2 to 12.2 kb or a set of high molecular weight markers ranging

in size from 8.3 to 48.5 kb. Both sets of DNA size standards were obtained from Bethesda Research Laboratories.

Procedures used in mating experiments:

(1) Matings in broth: Cells for mating were grown in 250-ml Erlenmeyer flasks containing 25 ml of BHI broth and incubated at 30°C with slow shaking. Donor and recipient strains were grown separately for 8 to 10 hours from 1% (v/v) transfers of 14- to 15-hour cultures. Each culture was diluted 1:50 in BHI broth, yielding 10^6 to 10^7 cells per ml, and mating mixtures were prepared by mixing 1 ml of donor cells with 1 ml of recipient cells in 20-mm culture tubes. Control tubes contained 1 ml of BHI broth and 1 ml of donor or recipient cells. Mixtures were incubated at 30°C with slow shaking. Samples were removed at times indicated and plated on appropriate selective media for determining the numbers of donors, recipients, and transcipients. Dilutions were made in peptone diluent. Plates were incubated at 30°C and colonies were scored after 24 to 48 hours.

When mating mixtures were prepared with streptomycin-resistant recipients and tetracycline-resistant donors, tetracycline-resistant transcipients were selected on L-agar containing streptomycin (200 µg/ml) and tetracycline (5 or 25 µg/ml). If the recipients were streptomycin-sensitive, tetracycline-resistant transcipients were selected on Min 1C agar supplemented with tetracycline and the appropriate growth requirement of the auxotrophic recipient. For selecting B. cereus transcipients 25 µg of tetracycline per ml was used, but with B. anthracis the number of transcipients recovered was greater when the concentration of tetracycline was only 5 µg per ml. Once transcipients were selected with the lower concentration of tetracycline, they were then fully resistant to 25 µg per ml. When recipients were rifampicin-resistant, rifampicin (10 µg/ml) was included in the selection medium.

Transfer frequency is expressed as the number of transcipients per ml divided by the number of donors per ml at the time of sampling. It should be emphasized that the use of both auxotrophic and drug-resistant strains allowed unambiguous strain selection and recognition.

(2) Matings on membranes: Donor and recipient cells were grown in 250-ml flasks containing 25 ml of BHI broth and incubated at 30°C on a reciprocal shaker, 80 excursions per min. Transfers (5%, v/v) from 14- to 16-hour cultures were grown for 5 hours. One ml of donor cells and 1 ml of recipient cells were mixed and 0.1-0.2-ml samples were spread onto Millipore DA or HA

membranes (Millipore Corp., Bedford, MA) which were placed on nonselective medium for 5 hr. BHI agar was usually used if the recipients were B. anthracis, B. cereus, or B. thuringiensis. PA agar was usually used for B. subtilis, and LG agar was used when the matings involved B. subtilis natto. To determine the number of donor and recipient cells per membrane, the mixture was diluted in peptone and plated on the appropriate selective media. Control mixtures contained 1 ml of BHI broth and 1 ml of donor or recipient cells. Plates were incubated at 30°C for 5 hours to allow mating and phenotypic expression. Membranes were subsequently transferred to agar plates containing tetracycline (for pBC16 transfer) and either rifampicin or streptomycin to select for recipients which had acquired the antibiotic resistance plasmid from the donor. To select for transfer of Tn917-containing plasmids, membranes were transferred to agar containing erythromycin and lincomycin, and either rifampicin or streptomycin. Colonies were scored after 1 to 2 days of incubation and transcipients were purified on the selective medium. The use of auxotrophically-marked strains facilitated unambiguous identification of transcipients. Frequency is expressed as the number of transcipients per donor.

Screening colonies for fertility. A replica plate mating technique was employed to screen large numbers of transcipients for fertility. Colonies of transcipients to be tested were picked to BHI agar to form master plates. These were incubated 16 to 18 h at 30°C and the colonies were replica plated to BHI agar plates that had been spread with 0.1 ml of spores (approx 1×10^8 CFU) of a recipient strain. The Str^r strains, B. anthracis UM44-2 and B. cereus UM20-1, and the Rif^r strain, Weybridge A UM23-4, were used as recipients. The plates were incubated 16 to 18 h at 30°C and the mixed growth was then replica plated to agar plates containing tetracycline and the appropriate antibiotic to select for the recipient strain. Incubation at 30°C was continued. After 16 to 20 h, patches of transcipient growth were present in areas corresponding to particular colonies on the master plate which were fertile.

Screening colonies for protective antigen production. Colonies were picked to plates of CA-agarose medium and incubated at 37°C in 20% CO_2 for about 16 hours. A zone of precipitate formed around colonies that produced the protective antigen component of anthrax toxin (12).

Electroporation of B. anthracis. Plasmids were introduced into B. anthracis Weybridge A UM23-1 and B. cereus 569 by electroporation using the method described by Bartkus and Leppla (1). An overnight culture was diluted 1:20 into BYJT (19% BHI, 0.5% yeast extract, 0.2% glucose, and 0.1 M Tris OH [pH 8]) and incubated with shaking (200 RPM) at 37°C for 1 h. The cells were harvested by centrifugation, washed 2 times in one-third volume of ice-cold electroporation buffer (0.625 M sucrose and 1 mM $MgCl_2$ [pH 4], filter sterilized), and resuspended in 1/30 the original culture volume of electroporation buffer. After the cell suspension was chilled on ice for 30 min, 0.8 ml was mixed with 1 to 10 µg of plasmid DNA in a cuvette with a 0.4-cm electrode gap. The mixture was then electroporated with a Gene Pulser (Bio-Rad Laboratories, Rockville Center, N.Y.) set at 2500 V and 25 µF capacitance. The cells were incubated on ice for 5-10 min after electroporation. The cells were then transferred to 2.2 ml of BYGT in a cotton-plugged 125-ml flask containing inducing concentrations of erythromycin and incubated at 30°C for 6 to 8 hrs. The cells were then plated on L agar plates containing selective levels of antibiotics. The plates were incubated at 30°C for 2 days.

Cryotransformation of Bacillus anthracis. The transposition selection vector pLTV3 was introduced into B. anthracis Weybridge A UM23-1 by cryotransformation using the method described by Stepanov, et al. (12). B. anthracis UM23-1 was grown overnight in 25 ml of BHI broth in a 250-ml cotton-plugged flask without shaking. Glycine and $MgCl_2$ were added to the overnight culture to a final concentration of 5% (w/v) and 0.05M, respectively. The incubation was continued at 37°C with slow shaking for 2 to 2.5 h. The cells were harvested by centrifugation and resuspended to a final density of 1×10^9 to 2×10^9 cells/ml in a medium containing 0.3% (w/v) Bacto peptone (Difco), 5% (w/v) glycine, 10% (w/v) polyethylene glycol 6000, and 0.1 M $MgCl_2$. Samples (150 µl) of cells were transferred to sterile glass vials and mixed with 50 µl of 0.1 M $MgCl_2$ and 50 µl of plasmid DNA. The samples were then frozen for 20 to 25 min at -12°C and thawed at 37°C. The mixture was transferred to 1 ml of BHI broth and incubated at 30°C with shaking for 4 h. Finally, the cells were plated on L agar plates containing selective levels of streptomycin (to select for the recipient strain) and selective levels of tetracycline (to select for recipient cells containing pLTV3). The plates were incubated at 30°C for 24 h.

Transposon mutagenesis in B. anthracis with the transposition selection vector pTV1. Approximately 5 x 10^8 spores of strains into which pTV1 had been introduced by electroporation or by transduction were inoculated into 25 ml of BHI-0.5% glycerol broth containing 15 µg of chloramphenicol (Cm) and 0.1 µg of erythromycin (Em) per ml (the latter to induce transposition) in a 250-ml cotton-plugged flask which was incubated at 30°C on a shaker (130 RPM). After 16 to 18 hours 1.0 ml was transferred to 25 ml of BHI-glycerol broth containing selective levels of Em (1 µg/ml) and lincomycin (Lm, 25 µg/ml) and no chloramphenicol. The transferred culture was incubated in a 43°C water bath with shaking (160 RPM) for 4 hours. The culture was transferred in a similar manner three additional times. Following the fourth transfer the culture was incubated for 12 to 16 hours, and then 1 ml was transferred to 25 ml of L broth (no antibiotics) and incubated on the shaker at 30°C until sporulation occurred as observed by phase microscopy (3 to 4 days). This last step was omitted when an asporogenous mutant was used.

Once sporulation had occurred, the culture was centrifuged at 10,000 RPM for 10 min at 4°C (Sorvall SS-34 rotor) and the spores were resuspended in 5 ml of sterile water. The resuspended spores were heat shocked (65°C, 30 min) and 0.1-ml samples of appropriate dilutions were spread on plates of L-agar containing 0.1 µg of Em per ml. The plates were incubated at 30°C for 24 hours.

Cells in which transposition of Tn917 and loss of pTV1 had occurred were identified by replica plating the colonies to L agar containing selective concentrations of erythromycin and lincomycin and to L agar containing 15 µg of chloramphenicol per ml. When spores were prepared following the mutagenesis procedure as described above, generally 95% or more were found to be transposants, i.e., Em^r Lm^r and Cm^s. To determine whether the transposon had inserted into a plasmid or the chromosome DNA-DNA hybridizations were carried out using ^{32}P-labelled Tn917 as the probe. Approximately 75% of the MLS-resistant, chloramphenicol-sensitive colonies isolated from transposon mutagenesis experiments with strains carrying pXO1 or pXO2 carried Tn917 on the plasmid.

Transposon mutagenesis of B. anthracis using the transposition selection vector pLTV3. Transposition of Tn917-LTV3 from pLTV3 to pXO1.1 was induced by growing B. anthracis UM23-1 etf5(pLTV3) in BHI broth with 0.1% (w/v) glycerol, containing 25 µg of tetracycline per ml to select for pLTV3-containing cells

and a subinhibitory concentration of erythromycin (0.1 μg/ml) to induce transposition. The cultures were incubated overnight at 30°C with shaking. To generate independent transposition events, several cultures were grown in individual cotton-plugged 20-mm tubes. The cells were cured of pLTV3 by serially transferring 1% of an overnight culture to BHI broth containing 0.1% glycerol and inhibitory concentrations of erythromycin and lincomycin. Four transfers were made and each was incubated for 12 h at 42°C with shaking.

The cultures were then transferred to L broth containing selective levels of erythromycin and lincomycin and allowed to sporulate (approximately 4 days). The spores were harvested and heat shocked (30 minutes at 65°C). The spore stock cultures were then diluted in peptone and spread onto L agar plates containing a subinhibitory concentration (0.1 μg/ml) of erythromycin. The plates were incubated at 30°C for 2 days and replica plated to L agar plates containing inhibitory concentrations of erythromycin (1 μg/ml) and lincomycin (25 μg/ml) and to L agar plates containing an inhibitory concentration (25 μg/ml) of tetracycline to screen for colonies cured of pLTV3. MLS^r Tc^s colonies were streaked for single colony isolation and examined for phenotypic alterations, gene fusions, and location of Tn917-LTV3 insertions.

RESULTS AND DISCUSSION

I. Physical and genetic analysis of the B. anthracis toxin plasmid, pXO1.1

Several phenotypes have been attributed to the presence of the 184-kb toxin plasmid, pXO1, in B. anthracis. Weybridge A strains cured of pXO1 exhibit changed phenotypes with respect to extent and rate of sporulation, sensitivity to bacteriophage, toxin production, growth on minimal medium, and colony morphology (14). One of our reasons for studying the genetics of pXO1 is to identify the regions of the plasmid responsible for conferring these phenotypic characteristics upon the host strain. Another reason for studying pXO1 is to identify other phenotypes which may be associated with the presence of pXO1 in B. anthracis. Following are descriptions of advances made during the past year in the physical and genetic analysis of pXO1. Most of these studies involved the use of Tn917-tagged plasmids.

Classification of pXO1 from different B. anthracis strains. As reported previously, Weybridge UM44 exhibited different plasmid-derived phenotypic characteristics from those observed in Weybridge A strains. (It should be recalled that the Trp⁻ auxotroph, Weybridge UM44, was isolated from the "wild-type" Weybridge strain, and Weybridge A was isolated from "wild-type" Weybridge as a mutant that grew much better than the parent strain on a minimal medium, minimal XO). The two strains, Weybridge UM44 and Weybridge A (and auxotrophs derived from the latter) differed in rate and extent of sporulation at 37°C, phage sensitivity, and growth characteristics on minimal medium. However, UM44-derived strains into which pXO1::Tn917 derivatives from UM23 were introduced showed characteristics similar to those typical of UM23. No apparent differences were observed in BamHI restriction patterns of pXO1 from Weybridge UM44 and pXO1 from Weybridge A UM23. However, there were some differences in the PstI and EcoRI restriction patterns of pXO1 from the two strains. Two new fragments were observed in PstI digests and in EcoRI digests of pXO1 from Weybridge UM44 compared to those of pXO1 from Weybridge A UM23. Because of differences observed in the toxin plasmid from the two strains, we have suggested that the plasmid of Weybridge UM44 be designated pXO1 and the one from Weybridge A strains be designated pXO1.1. Similarly, the toxin plasmid from other B. anthracis strains will be designated pXO1.2, pXO1.3, etc., as they are characterized.

As described in the Annual Report of 1989, our laboratory isolated several insertional mutants which were generated by transposon mutagenesis of pXO1.1. Deletion analysis has also been employed to study the genetics of this toxin plasmid. Several transposants and deletants have been isolated which exhibit altered phenotypes with respect to extent and rate of sporulation, sensitivity to bacteriophage, and toxin production, phenotypes which have been attributed to the presence of pXO1.1. Restriction analysis has revealed regions of the plasmid which may be involved in conferring some of these phenotypic characteristics.

This section of the report describes the advances made this year in the physical and genetic analysis of pXO1.1. Furthermore, advances have been made in isolating mutants involved in the regulation of the production of the toxin components.

Location of the toxin structural genes on pXO1.1 and pXO1. The restriction map of pXO1 constructed by D. Robertson (11) has been used as a

reference for determining the location of the Tn917 insertions within pXO1.1 with respect to the location of the structural genes of the toxin components, PA, LF, and EF. To confirm that the restriction fragments containing the toxin structural genes on pXO1.1 correspond to those fragments on Robertson's map, ^{32}P-labelled plasmid vectors, pPA101, pLF7, and pSE42, each containing one of the toxin structural genes, were used in DNA-DNA hybridization studies to probe BamHI-, PstI-, or EcoRI-digested pXO1.1 and pXO1.

The fragments of pXO1.1 to which each of the probes had hybridized are listed in Table 2. The same results were observed in hybridization analysis of BamHI-, PstI-, or EcoRI-digested pXO1 (data not shown). In summary, pPA101, which contains the structural gene for PA, hybridized to the 6.0-kb BamHI fragment, the 6.0-kb and 18.1-kb PstI fragments, and the 6.2-kb and 5.6-kb EcoRI fragments. pLF7, which contains the LF structural gene, hybridized to the 34.8-kb BamHI fragment, the 6.6-kb and 6.0-kb PstI fragments, and the 5.8-kb, 5.6-kb, and 0.7-kb EcoRI fragments. Finally, pSE42, which contains the EF structural gene, hybridized to the 7.4-kb BamHI fragment, the 18.1-kb and 5.5-kb PstI fragments, and the 8.0-kb, 3.1-kb, and 1.1-kb EcoRI fragments. The plasmids containing the structural genes for PA, LF, and EF hybridized to the BamHI and PstI fragments corresponding to similar fragments depicted in Robertson's map of pXO1. The 34.8-kb BamHI fragment, which contains lef, corresponded to the 29-kb BamHI fragment on Robertson's map.

Restriction analysis of the 34.8-kb BamHI fragment from pXO1.1. PstI-digested pXO1.1 was probed with the 34.8-kb BamHI fragment to determine the PstI profile of this fragment. The probe was obtained by digesting pXO1.1 with BamHI, eluting the 34.8-kb fragment, and radiolabelling the fragment with ^{32}P-dGTP for use in DNA-DNA hybridization with the PstI-digested pXO1.1. Preliminary data showed that six PstI fragments (5.2-kb, 6.0-kb, 6.3-kb, 6.6-kb, 9.3-kb, and 18.1-kb) shared homology with the 34.8-kb BamHI fragment. Further analysis will be necessary to arrange these PstI fragments within the 34.8-kb BamHI fragment. Since a majority of the deletion derivatives isolated contain deletions within this fragment, mapping of the 34.8-kb BamHI fragment will be useful in further defining the deletions within this region. Some of the results from the PstI restriction data conflicted with Robertson's PstI restriction map of the 29-kb BamHI fragment. One reason for the difference observed between the restriction profiles may be differences in pXO1 plasmids isolated from different B. anthracis strains.

Analysis of the 44.2-kb BamHI fragment from UM23 tp21. The Tn917-tagged pXO1.1 derivative isolated from Weybridge A UM23 tp21 contained deletions and/or alterations in more than one BamHI fragment (6.0-kb, 13.9-kb, and 34.8-kb). The resulting phenotype was PA^- LF^+ EF^+, suggesting that this plasmid did not contain the structural gene for PA. Restriction analysis has shown that the fragments carrying the LF and EF structural genes are present. The BamHI restriction profile of this plasmid also showed that this deletion derivative contained a new 44.2-kb fragment. As determined by hybridization with the 34.8-kb BamHI fragment isolated from wild-type pXO1.1 and by hybridization with Tn917, the 44.2-kb BamHI fragment contained the 34.8-kb BamHI or portions of this fragment and Tn917. To determine what other fragment(s) have joined the 34.8-kb BamHI fragment and the 5.2-kb Tn917 to form the 44.2-kb fragment, BamHI-digested wild-type pXO1.1 was probed with the 44.2-kb BamHI fragment which had been labelled with ^{32}P-dGTP. This fragment hybridized to the 34.8-kb and the 13.9-kb BamHI fragments of wild-type pXO1.1. The results from these DNA-DNA hybridization studies, as well as the previous results from BamHI and PstI restriction experiments, indicate that the 34.8-kb and the 13.9-kb BamHI fragments contains deletions. These data also suggest that the 6.0-kb BamHI fragment is located between these two fragments (similar to Robertson's map) and that this region incurred deletions upon Tn917 insertion, thus resulting in the joining of the 13.9-kb and the 34.8-kb fragments.

Isolation of more Tn917-tagged pXO1.1 derivatives. As reported in the annual report of 1989, insertions of Tn917 from the temperature-sensitive transpostion selection vector pTV1 into pXO1.1 were generated by erythromycin-induced transposon mutagenesis. The cells were subsequently cured of pTV1 by serial passage at 43°C. Our initial approach was to isolate random MLS-resistant, choroamphenicol-sensitive colonies, confirm Tn917 insertions into pXO1.1 by DNA-DNA hybridizations using ^{32}P-labelled Tn917 as the probe, and finally, determine phenotypic alterations.

The approach we took during the past year was to isolate MLS-resistant, chloramphenicol-sensitive colonies altered in particular plasmid-derived phenotypes. MLS-resistant, chloramphenicol-sensitive colonies were isolated from spore stocks obtained from previous transposon mutagenesis experiments. The colonies were screened for protective antigen (PA) production on halo immunoassay plates incubated in 20% CO_2 or in air (bicarbonate was omitted

from those plates incubated in air). These colonies were also screened for sensitivity to CP-51 by picking colonies to phage assay agar plates spread with 10^8 to 10^9 PFU and looking for plaque formation within the colony. Thirty-five colonies which appeared to be altered in these specific phenotypes were isolated and twenty-one of these transposants were shown to contain Tn917 within pXO1.1 as determined by DNA-DNA hybridizations using ^{32}P-labelled pTV1 as the probe.

Table 3 lists the phenotypes tested and the results obtained from these experiments. Twelve of these transposants did not produce PA as determined by the halo immunoassay test. One PA^+ transposant, tp62, formed a halo on immunoassay plates incubated either in CO_2 or in air. This strain was tested for the production of PA in Casamino acids (CA) broth. As will be discussed in greater detail below, tp62 produced more PA both in the presence and the absence of added bicarbonate than the parent strain, UM23. These results suggest that this mutant overproduces PA and doesn't require added bicarbonate for production of PA.

Two transposants, tp49 and tp71, exhibited deletions within pXO1.1::Tn917 of greater than 100 kb. As determined by the halo immunoassay, tp49 and tp71 are PA^+ and PA^-, respectively. These transposants also showed an increase in the extent of sporulation at 37°C as compared to the parent strain, UM23. The number of spores observed in these transposants by phase-contrast microscopy, though, did not appear to be as numerous as in the parent strain cured of pXO1.1, UM23C1. UM23 tp47 and tp64 exhibited an increase in the extent of sporulation; however, the number of spores observed was less than that observed for tp49 and tp71, Several transposants appeared to be asporogenous at 37°C as observed by phase-contrast microscopy; however, further analysis will be necessary to confirm this phenotype. In particular, tp62 which produced an increased amount of PA appeared to be asporogenous at 37°C and oligosporogenous at 30°C. UM23 sporulates extensively at 30°C and is oligosporogenous at 37°C. In comparison to UM23, tp62 appears to exhibit an alteration in the extent of sporulation. Whether the alterations in the extent of sporulation in all cases is caused by Tn917 insertion within pXO1.1 or is chromosomally related remains to be determined.

Three transposants exhibited an alteration in the sensitivity to bacteriophage. CP-51 formed very small plaques on tp49 and tp71, and larger but more turbid plaques on tp65. The plaques observed on tp65 were similar to

those observed on tp28. UM32 tp28 had been isolated previously from these transposon mutagenesis experiments and was shown to exhibit an alteration in sensitivity to CP-51. The insertions which occurred in UM23 tp28 and tp65 were from independent transposition events.

Tests for production of protective antigen by UM23 tp62. As previously stated, tp62, which contains Tn917 in pXO1.1, produced more protective antigen in the presence and the absence of added bicarbonate than the parent strain, UM23. The phenotype observed for tp62 was designated as PA^{a+} (PA production in air).

To optimize the conditions under which tp62 will produce PA, tp62, as well as UM23, were grown in CA broth under various conditions. Cells of the strain to be tested were grown overnight at 37°C with shaking in CA broth without bicarbonate. Selective levels of erythromycin and lincomycin were added to the medium for UM23 tp62 cultures and an additional 40 µg of uracil per ml was added since Weybridge A UM23 and its derivatives are Ura^-. One-tenth ml of the overnight culture was transferred to 100 ml of buffered CA broth (pH 6.5, 7.0, 7.5, 8.0, or 8.5) supplemented as above and including 0.2% Norit A, or to 100 ml of CA broth supplemented as above and including 0.2% Norit A and 0.72% $NaHCO_3$. The cultures were grown at 37°C in 250-ml cotton-plugged flasks either statically or with slow shaking (100 RPM).

Ten-ml samples of each culture were removed at various times, mixed with 0.5 ml of horse serum, and filtered through Millipore HA membranes without prefilters. The culture filtrates were diluted by serial two-fold dilutions and tested for PA antigen by an Ouchterlony double diffusion assay using goat antiserum prepared against B. anthracis (supplied by USAMRIID). The plates were incubated at 37°C with high humidity and read after 24 and 48 hr. The highest dilution which produced a visible line of precipitation was taken as the end point. The identity of PA in the filtrates was determined by observing the pattern of lines that formed between the sample filtrate and an adjacent well containing PA which was isolated from filtrates of Weybridge A UM23 grown under conditions known to be favorable for PA production.

As shown in Table 4, tp62 produced considerably more PA than UM23 when grown statically in buffered CA broth at pH values of 7.5, 8.0, and 8.5 without added bicarbonate and in static CA broth containing bicarbonate. In the presence of bicarbonate tp62 also produced more PA than UM23 but the differences in yields were not as dramatic. Table 5 shows the results

obtained with cultures grown in CA broth in shaken flasks. UM23 tp62 produced a maximum amount of PA in buffered CA broth without bicarbonate at pH 7.5 and pH 8.0 under shaken conditions. The yields of PA from tp62 were considerably greater than those produced by UM23 under the same conditions. For reasons unknown, tp62 did not grow well in shaken flasks of CA broth containing bicarbonate. T. Koehler in J. Collier's laboratory at Harvard University also tested tp62 for production of PA and LF in BHI broth and in RM medium. She found that tp62 did not grow well in RM medium. However, tp62 produced PA and LF in BHI broth in the absence of bicarbonate, whereas Weybridge A UM23 did not produce detectable amounts of PA or LF under the same conditions. We conclude from the results of all these experiments that added bicarbonate is not essential for PA production by tp62, but rather, the pH of the medium is a more critical factor.

Transfer of pXO1.1::Tn917 from Weybridge A UM23 tp62 to B. anthracis strains cured of pXO1. As stated above, tp62, which produces more PA both in the presence and absence of added bicarbonate than the parent strain, UM23, also appears to sporulate less frequently than UM23 at 37°C and at 30°C. To determine whether these phenotypes are plasmid-associated, the Tn917-tagged pXO1.1 derivative from tp62 was transferred by conjugation to B. anthracis strains cured of pXO1.

The B. thuringiensis fertility plasmid pXO12, tagged with Tn917, was introduced into tp62 by conjugation. A transcipient, tp62 tr2, containing pXO12::Tn917 was then used in a second mating to transfer pXO1.1::Tn917 to Weybridge UM44-1C9 (Ind^- Str^r $pXO1^-$) and to Weybridge A UM23C1-2 (Ura^- Rif^r $pXO1^-$). Transcipients were selected directly for the transfer of pXO1.1::Tn917 on L agar plates containing inhibitory concentrations of erythromycin and lincomycin and also containing inhibitory concentrations of streptomycin or rifampicin (depending on the recipient that was used). Str^r MLS^r colonies (UM44-1C9 transcipients) or Rif^r MLS^r colonies (UM23C1-2 transcipients) were then tested for PA production on halo immunoassay plates. Approximately 50% of the UM44-1C9 transcipients and 20% of the UM23C1-2 transcipients tested were PA^+. Four colonies were isolated from each strain (UM44-1C9 tr1 to tr4 and UM23C1-2 tr1 to tr4). These transcipients were then examined for phenotypic alterations. Transcipients examined for PA production were grown in buffered CA broth (pH 7.5) without bicarbonate in shaken flasks

at 100 RPM for 15 h. They were also grown statically in CA broth containing added bicarbonate for 27 h (normal growth conditions for PA production).

Results (not shown) indicated that the UM44-1C9 transcipients and the UM23C1-2 transcipients produced about the same amount of PA as tp62 and more than UM23 under both sets of growth conditions. The sporulation pattern of the transcipients at 30°C and at 37°C appeared to be similar to that of tp62. These results indicate that the altered phenotypes observed in tp62 are plasmid-associated.

Restriction analysis of Tn917-tagged pXO1.1 derivatives from transposants which may be altered in regulation of toxin production. Preliminary restriction analysis of pXO1.1::Tn917 isolated from tp62 showed that the 34.8-kb BamHI fragment was altered. More precisely, the 9.3-kb PstI fragment which is nested within the 34.8-kb BamHI fragment has been altered by Tn917 insertion. The new BamHI fragment generated from this Tn917-tagged plasmid was about 38.7 kb in size and the new PstI fragment generated was about 14.6 kb. As there are no sites within Tn917 for restriction by BamHI or PstI, the new fragment generated by either restriction enzyme is most likely the result of Tn917 insertion into the fragment that is regarded as altered. Tables 6 and 7 show the altered phenotype of tp62 and the BamHI restriction pattern of pXO1.1::Tn917 which was isolated from tp62. These results suggest that sequences within the 34.8-kb BamHI fragment may be involved in regulation of PA or toxin production. They also suggest that this same region may be involved in regulation of sporulation.

Previously we had suggested that a region may exist in the 13.9-kb BamHI fragment which plays a role in the regulation of toxin production. As shown in Tables 6 and 7, five transposants, tp18, tp26, tp29, tp32, and tp38, which exhibit an altered 13.9-kb BamHI fragment, did not produce PA. Two of these PA^- mutants (tp29 and tp32) were analyzed by S. Leppla at USAMRIID and were found to be deficient in synthesis of the other toxin components, EF and LF. UM23 tp29 was EF^- $LF^{+/-}$ and tp32 was EF^+ $LF^{+/-}$. The other transposants (tp18, tp26, and tp38) have not been analyzed for EF and LF production. All of the Tn917-tagged pXO1.1 derivatives isolated from these PA^- mutants exhibited a new 19.5-kb BamHI fragment. The 19.5-kb BamHI fragments from tp29 and tp32 were shown to contain Tn917 by DNA-DNA hybridizations using ^{32}P-labelled Tn917 as the probe. Not all insertions within this region affected PA (or toxin) production. The pXO1.1::Tn917 derivative isolated from tp27 showed that Tn917

had also inserted into the 13.9-kb BamHI fragment (to give a 19.5-kb fragment); however, tp27 synthesized PA, LF, and EF. Data obtained from EcoRI digestion of the Tn917-tagged pXO1.1 derivatives from tp27, tp29, and tp32 showed that Tn917 had inserted into different regions of the 13.9-kb BamHI fragment, thus accounting for the differences in the observed phenotypes. The Tn917-tagged pXO1.1 derivative from the PA^+ strain, tp27, was altered in the 5.8-kb EcoRI fragment, while pXO1.1::Tn917 isolated from tp29 and tp32 was altered in the 8.0-kb EcoRI fragment. These results are consistent with the idea that a region of the 13.9-kb BamHI fragment may be involved in regulation of toxin production.

Generation of gene fusions in UM23 tp62. To generate in vivo lacZ gene fusions within pXO1.1 and determine whether Tn917 had inserted into a gene or a site involved in regulation, we carried out experiments to replace existing Tn917 insertions within pXO1.1 with Tn917 cat lac from pTV53 by homologous recombination. The transposon in pTV53 carries a promoterless lacZ gene and a promoterless cat-86 gene in tandem. If the original Tn917 insertion was downstream of a promoter, then cells in which the Tn917 in pXO1.1 was replaced with Tn917 cat lac in the right orientation should express chloramphenicol resistance. pTV53 was transduced from UM23-1 etf3(pTV53) into tp62. Instead of selecting for transductants containing pTV53, we selected transductants containing Tn917 cat lac gene fusions on L agar containing inhibitory concentrations of chloramphenicol.

Three Cm^r transductants were isolated. Plasmid profiles showed that these colonies (designated tp62 td1, td2, and td3) contained pTV53 along with the pXO1.1::Tn917 derivative from tp62. To eliminate the possibility that read-through of the transposon within pTV53 was the factor contributing to chloramphenicol resistance, tp62 td2 was cured of pTV53 by serial transfers at 43°C in the presence of inhibitory concentrations of chloramphenicol. Cells that are cured of pTV53 are sensitive to tetracycline. The plasmid profiles of three Cm^r Tc^s colonies (designated as tp62 td2C1, td2C3, and td2C4) showed that pTV53 was not present. To show that the Cm^r phenotype was due to expression of a gene fusion and not to spontaneous mutation, the colonies were tested for beta-galactosidase activity which would provide evidence that a lacZ fusion had occurred. This was achieved by either picking the colonies to L agar containing 30 ug of X-gal/ml or by spraying the plates with approximately 0.2 ml of a 10 mg/ml solution of methylumbelliferyl-beta-D-

galactoside (MUG). Colonies which express beta-galactosidase activity turn blue on X-gal plates and fluoresce under long wavelength ultraviolet light when sprayed with MUG. The cured colonies exhibited beta-galactosidase activity when tested with the galactoside indicator substrates. These results suggested that Tn*917* *cat* *lac* either replaced the existing Tn*917* insertion, or transposed to a different region on the plasmid or chromosome. Restriction analysis of the plasmids from these cured strains will be used to determine the location of the Tn*917* derivative.

Analysis of UM23 tp49 and UM23 tp71. As discussed previously, two transposants were isolated which exhibited deletions within pXO1.1::Tn*917* of greater than 100 kb. UM23 tp49 and tp71 were determined by halo immunoassay to be PA^+ and PA^-, respectively. When these cultures were examined for the production of PA in CA broth containing bicarbonate, tp49 appeared to produce approximately two times more PA than the parent strain, UM23. Under similar conditions tp71 did not produce any detectable amounts of PA.

Table 8 lists the *Bam*HI and the *Pst*I fragments found in the plasmids from tp49 and tp71. These deletion derivatives are missing many of the fragments normally generated from *Bam*HI or *Pst*I digestion of wild-type pXO1.1. *Bam*HI appears to linearize the plasmid from tp49. One of the *Bam*HI fragments generated from the deletion derivative of tp71 migrated to the same position as the 13.9-kb *Bam*HI fragment generated from pXO1.1. As will be discussed later, these two *Bam*HI fragments may not be related. *Pst*I fragments of *ca.* 12.9 kb and *ca.* 16.0 kb were generated from the deletion derivatives obtained from tp49 and tp71, respectively, which do not correspond to any *Pst*I fragments obtained from wild-type pXO1.1. The sizes of the pXO1.1::Tn*917* deletion derivatives from tp49 and tp71 were found, on the average, to be approximately 40.0 kb and 47.5 kb, respectively. These sizes were determined from the summation of the *Bam*HI or *Pst*I restriction fragments. Results from the restriction analysis also showed that tp49 may contain the structural genes for PA and LF, while tp71 may contain only the structural genes for LF.

DNA-DNA hybridizations were used to determine what toxin structural genes were present on these deletion derivatives and to determine the location of Tn*917*. The restriction fragments to which each of these probes hybridized are shown in Table 9. Briefly, *Bam*HI- and *Pst*I-digested plasmid DNA obtained from tp49 and tp71 were probed with ^{32}P-labelled pPA101, pLF7, pSE42, or Tn*917*. Tn*917* was found to be located on the *ca.* 40.0-kb *Bam*HI fragment or,

more precisely, on the ca. 12.9-kb PstI fragment of the plasmid from tp49. The location of Tn917 on the plasmid from tp71 was on the ca. 16.0-kb PstI fragment or, more precisely, on the 13.9-kb BamHI fragment. As stated earlier, this BamHI fragment migrates to the same position as the 13.9-kb BamHI fragment from wild-type pXO1.1. The BamHI fragment from which the 13.9-kb BamHI fragment from the deletion derivative of tp71 originated cannot be determined from these data.

The plasmid vector pPA101 containing pag hybridized to the ca. 40.0-kb BamHI fragment or, more precisely, to the ca. 12.9-kb and the 6.0-kb PstI fragments indicating that the Tn917-tagged plasmid from tp49 does contain the PA structural genes, thus confirming the PA^+ phenotype. The common PstI fragment from pXO1.1 and from the deletion derivative of tp49 to which pPA101 hybridized was the 6.0-kb fragment. These results suggested that the ca. 12.9-kb PstI fragment was a deletion of the 18.1-kb PstI fragment (the fragment in wild-type pXO1.1 to which pPA101 hybridized). The hybridizations also revealed that the deletion derivative from tp49 contained the structural genes for LF on the 6.6-kb and 6.0-kb PstI fragments which correspond to the fragments from wild-type pXO1.1.

The plasmid vector pPA101 did not hybridize to any fragments from tp71 consistent with the PA^- phenotype. The plasmid from tp71 appears to contain the structural genes for LF; pLF7 hybridized to the ca. 16.0-kb and the 6.6-kb PstI fragment and to the 34.8-kb BamHI fragment. Finally, the data obtained from hybridization of the deletion derivatives from both tp49 and tp71 with pSE42 (which contains EF structural genes) suggested that neither plasmid contained the structural genes for EF. These transposants, tp49 and tp71, will need to be analyzed for the production of LF and EF to confirm these results.

Characterization of UM23C1 tds1(pXO1.1::Tn917Δ1). The Tn917-tagged pXO1.1 deletion derivative of UM23C1 tds1 was obtained by transducing the tagged derivative from UM23 tp2A to UM23C1 ($pXO1.1^-$) with CP-51. As reported in the Annual Report of 1989, the size of the plasmid from tds1 is approximately 81.1 kb. UM23C1 tds1 showed alterations in toxin production (PA^-, EF^-, LF^-); however, the phenotypes with respect to sporulation and sensitivity to CP-51 were similar to those of strains containing pXO1.1. Since this plasmid has a large part of the original DNA deleted, yet confers some characteristics on B. anthracis similar to those observed with pXO1.1-

containing strains, it must have retained the region(s) involved in expression of these phenotypes (i.e. sporulation and CP-51 sensitivity). To confirm this, tds1 was cured of the Tn917-tagged pXO1.1 deletion derivative by serial passage at 43°C. The cured colonies were selected on the basis of loss of MLS-resistance. Plasmid extractions from MLS-sensitive colonies showed the loss of the 81.1-kb plasmid. Two colonies were isolated (designated tds1C1 and tds1C2) and were tested for phenotypic alterations. As shown in Table 10, these strains exhibited characteristics (i.e. sporulation and sensitivity to CP-51) similar to those of UM23 strains cured of pXO1.1. The 81.1-kb plasmid was also transduced to UM44-1C9 by CP-51-mediated transduction. MLS-resistant colonies were isolated and tested for altered plasmid-derived phenotypes. Normally UM44-1 (containing pXO1) sporulates well at 37°C and exhibits plaque formation when infected with CP-51. As shown in Table 10, sporulation and sensitivity to CP-51 in UM44-1C9 td13 and td14 diminished significantly and resembled characteristics similar to those of pXO1.1-containing strains. The results obtained from these two experiments provided evidence that the observed characteristics are plasmid-associated. Further analysis will be necessary to locate the regions associated with these phenotypes.

Introduction of pLTV3 into B. anthracis by electroporation and cryotransformation. The transposition selection vector, pLTV3, was constructed by Camilli, et al. (4). The plasmid contains a Tn917 derivative, Tn917-LTV3, which carries a promoterless lacZ gene as well as the erm gene, a cat gene, and genes for cloning in E. coli. The plasmid itself is 22.1 kb in size and carries the gene for tetracycline resistance. The purpose for introducing pLTV3 into B. anthracis is to generate more transposon-tagged pXO1.1 derivatives by transposon mutagenesis, thus, increasing our library of insertional mutants and hopefully, creating in vivo lacZ gene fusions. The transposon will also be used to replace simple Tn917 insertions in existing pXO1.1::Tn917 derivatives by selecting for chloramphenicol resistance. These "recombinants" can then be screened for transposon replacement and for gene fusions.

pLTV3 was isolated from B. subtilis PY1178 and introduced into Weybridge A UM23-1 by electroporation using the method described by Bartkus and Leppla (1) or by cryotransformation using the method described by Stepanov, et al. (12). On the average, approximately 2×10^4 transformants per ml were obtained in two electroporation experiments and 20 transformants per ml were

obtained from the cryotransformation experiment. Strains designated UM23-1 etf5 and etf6 were derived from independent electroporation experiments and were shown to contain pLTV3 and pXO1.1. The strain designated UM23-1 ctf1 was isolated from the cryotransformation experiment and was also shown to contain pLTV3 and pXO1.1.

Generation of Tn917-LTV3-tagged pXO1.1 derivatives. Insertions of Tn917-LTV3 into pXO1.1 from pLTV3 were generated as described in the Materials and Methods section of this report. In 7 out of 8 independent transposon mutagenesis experiments, approximately 100% of the colonies were MLS-resistant and tetracycline-sensitive indicating that Tn917-LTV3 had transposed either to pXO1.1 or to the chromosome and that the cells were cured of pLTV3. MLS^r Tc^s colonies were screened for protective antigen production by picking colonies to halo immunoassay agar plates, for gene fusions by testing for beta-galactosidase activity using MUG as the indicator substrate, and for sensitivity to CP-51 by looking for plaque formation within colonies which had been picked to phage assay agar plates spread with 10^8 to 10^9 PFU. Several transposants have been isolated which exhibit beta-galactosidase activity and are either PA^+ or PA^- as determined by formation of a halo on the immunoassay plates. Only one colony has been isolated so far which may be sensitive to bacteriophage, and it also appears to be PA^-. The plasmid profile of this strain revealed that the plasmid is a deletion derivative of pXO1.1 having more than 100 kb missing. DNA-DNA hybridizations will be employed to determine the location of Tn917-LTV3 in these strains.

II. TRANSPOSON MUTAGENESIS OF THE B. ANTHRACIS CAPSULE PLASMID, pXO2, AND CHARACTERIZATION OF THE INSERTION MUTANTS

The B. anthracis plasmid pXO2 carries information for synthesis of a D-glutamyl polypeptide capsule. Capsule formation is the only function that has thus far been attributed to this plasmid. B. anthracis strains harboring wild-type pXO2 require CO_2 and bicarbonate for capsule synthesis. When such strains are grown in the presence of CO_2 and bicarbonate the colonies appear very mucoid; in the absence of CO_2 and bicarbonate the colonies appear rough.

In previous studies B. anthracis strains harboring pXO2 were divided into 3 groups with respect to capsule phenotype: (1) Strains that produce capsules only when grown on media containing bicarbonate and incubated in a

CO_2-rich atmosphere (Cap^{c+} phenotype); (2) Strains that produce capsules when grown in air in the absence of added bicarbonate or CO_2 (Cap^{a+} phenotype); and (3) Strains that are noncapsulated under all growth conditions and yet retain pXO2 (Cap^- phenotype). Spontaneous mutations resulting in the second and third phenotypes hav. been shown to be associated with pXO2.

Research in th s area during the past year has focused primarily on isolation, identification, and characterization of mutants resulting from Tn917 insertions in B. anthracis 4229 UM12 Nal^r. A number of mutants having very interesting phenotypes have been isolated and some of them are described here.

Introduction of pTV1ts into B. anthracis 4229 harboring the capsule plasmid pXO2. The temperature-sensitive transposition selection vector pTV1ts was introduced into B. anthracis 4229 UM12 Nal^r by CP-51-mediated transduction. CP-51 propagated on Weybridge A UM23C1 td25(pTV1ts) transferred pTV1ts at frequencies high enough to recover transductants. Plasmid extracts of representative Cm^r MLS^r transductants were analyzed by gel electrophoresis to screen for the presence of pXO2 and pTV1. Transductants confirmed to contain both plasmids were then used to generate Tn917 insertions in pXO2.

Isolation of Tn917-tagged pXO2 derivatives. Cells containing pTV1ts and pXO2 were induced for transposition of Tn917 in the presence of subinhibitory levels of erythromycin (0.1 µg/ml) and then cured of pTV1 by several passages of the cells at 43°C (non-permissive temperature for replication of the plasmid vector) while maintaining selection for MLS resistance. After four transfers cells were allowed to sporulate in L broth or NBYMn broth at 30°C for about 4 days. After sporulation had occurred, the cultures were centrifuged, the spores were resuspended in 5 ml of sterile water and finally heat-shocked (30 min at 65°C) to kill any remaining cells. Spore suspensions were diluted and plated on LE agar (L agar with 0.1 µg of erythromycin per ml) for isolated colonies. Single colonies were screened for sensitivity to chloramphenicol and resistance to erythromycin and lincomycin to confirm loss of pTV1 and retention of Tn917. A total of 8,320 colonies from four experiments were screened in this manner Transposition had occurred in about 99% of the colonies. The presence of Tn917 in pXO2 or the chromosome was confirmed by DNA-DNA hybridization experiments using ^{32}P-labelled Tn917 DNA as the probe. Approximately 64% of the MLS-resistant chloramphenicol-sensitive colonies carried Tn917 in pXO2.

Isolation of Cap^{a+} transposants by utilizing phage CP-54. Another approach was also taken to isolate transposants which have the ability to produce capsules in the absence of added CO_2 and bicarbonate. BHI broth was inoculated with a loop of spore stock that had been prepared directly from a B. anthracis 4229 UM12(pXO2, pTV1ts) culture following the induction and curing procedures. This culture was grown for 16 to 18 hours and 0.1 ml was spread in duplicate on PAEL agar (PA agar with inhibitory concentrations of erythromycin and lincomycin) and incubated at 37°C in air. Following 2 to 3 hours of incubation, a set of plates was spread with phage CP-54 and then incubated at 37°C in air. CP-54 is more lytic than CP-51 and it can not adsorb to encapsulated cells, allowing selection for encapsulated cells. Mucoid colonies appeared after two days of incubation both on plates challenged with CP-54 and on control plates that contained no phage. The fact that mutants which produced capsules in air occurred at a frequency high enough to be detected without exposure to phage suggests that insertion(s) of Tn917 caused the mutation(s) that relieved the CO_2 and bicarbonate requirement. It has been our experience that spontaneous mutants which do not require CO_2 or bicarbonate for capsule synthesis occur at frequencies too low to permit their detection without selection with bacteriophage.

Phenotypes of transposants. MLS^r Cm^s colonies, which were presumed to be transposants cured of pTV1, were observed for capsule phenotypes, Cap^{c+}, Cap^{a+}, or Cap$^-$. Isolated colonies were picked to LPA and LPACO_3 agar containing inhibitory concentrations of erythromycin and lincomycin and incubated at 37°C with and without CO_2. Transposants exhibited phenotypic differences in capsule production that could be categorized as: (1) Noncapsulated strains (Cap$^-$) which retained pXO2 but produced rough colonies under all growth conditions. (2) Strains that produced capsules only when grown in media containing bicarbonate and incubated in a CO_2-rich atmosphere (Cap^{c+}). These appeared to be similar to wild-type pXO2-containing strains with the exception of a few which did not grow when incubated in air and a few which appeared to be polypeptide overproducers, resembling Bacillus licheniformis in this respect. (3) Strains that produced capsules when grown in air in the absence of bicarbonate (Cap^{a+}). Some of these synthesized capsules both in the presence and absence of CO_2 and a few did not grow in the presence of 20% CO_2. From these experiments a series of colonies were isolated to give 45 transposants which exhibit the different phenotypes

described above and of which at least 30 represent independent transposition events. These transposants and their phenotypes are listed in Table 11.

Among the 45 confirmed transposants the majority showed capsule synthesis only in the presence of bicarbonate and CO_2, a few of them did not form capsules in the presence or absence of bicarbonate and CO_2, and a few of them produced capsules in air without added bicarbonate or CO_2. pXO2 in most of the transposants appeared to be of wild-type size. In some instances pXO2::Tn*917* appeared to be larger than wild type pXO2, and a few transposants contained deleted versions of pXO2::Tn*917*.

The pXO2::Tn*917* derivatives in some of the transposants that have the Cap^{a+} phenotype have been observed to undergo deletions; deletions appear to occur more frequently in such strains when they are grown in 20% CO_2. We now have a collection of strains carrying deleted versions of pXO2::Tn*917*. Some of these are Cap^+ and others are Cap^-. One of them, tp24-17, is very small (probably less than 12 kb) but its host cells are still capable of producing capsules. However, they appear to produce less capsular material than strains carrying wild-type pXO2, suggesting that the deleted form of the plasmid retains the structural genes for capsule synthesis but requires an additional determinant to enhance encapsulation.

As mentioned previously, some transposants appear to be polypeptide overproducers. The "stringy" properties of their colonies and their propensity to produce confluent growth (mixture of cells and polypeptide) resemble characteristics of *B. licheniformis* with respect to polypeptide production. Upon continued incubation the growth on agar plates loses its mucoid characteristic and appears rough and "dry". This is characteristic of *B. licheniformis*. Examples of transposants having this phenotype are 4229 UM12 tp49 and tp50. Hybridization studies with these two transposants have shown that in tp49 Tn*917* is inserted into the plasmid and in tp50 Tn*917* is inserted into the chromosome. The observation that colonies of these transposants appear rough and "dry" after a relatively short period of time might suggest production of a D-glutamyl polypeptidase. Such an enzyme has been shown to occur in *B. licheniformis*, but to our knowledge there has been no report of a similar enzyme in *B. anthracis*. It is interesting that the two transposants which have similar phenotypes have Tn*917* in different locations, on pXO2 in tp49 and on the chromosome in tp50. To determine whether the

insertion of Tn917 is responsible for the unusual properties of these two transposants, we carried out the following experiments.

Analysis of 4229 UM12 tp49 and tp50 by CP-51 mediated transduction. To provide evidence that the mutant phenotypes of 4229 UM12 tp49 and tp50 are the result of Tn917 insertions rather than spontaneous mutations, transductions were performed. Phage CP-51 was propagated on each of the transposants to prepare lysates which were then used to transduce MLS resistance into appropriate strains. Cells used as recipients for transduction of pXO2::Tn917 from tp49 were streptomycin resistant and cured of pXO2. Str^r MLS^r transductants were screened for capsule formation. Cells used as recipients for transduction of the chromosomally inserted Tn917 were Str^r and they contained wild-type pXO2. Str^r MLS^r transductants were screened for capsule formation.

The results show that in all instances MLS^r transductants exhibited the specific donor phenotype. To confirm the presence and location of Tn917 in the transductants we carried out DNA-DNA hybridization experiments using ^{32}P-labelled pTV1 DNA as the probe. Results showed that when the donor was tp49 carrying pXO2::Tn917, the transposon was located on pXO2 in the transductants. Transductants which received MLS resistance from chromosomally-marked tp50 did not show Tn917 on pXO2; however, location in the chromosome was not clear. This hybridization test is being repeated.

To gather further evidence for the location of Tn917 in the transductants referred to above, we looked for spontaneous Cap^- mutants that had lost pXO2 and tested them for MLS resistance. If a transductant carried pXO2::Tn917, as would be expected when tp49 was the donor, then spontaneous Cap^- mutants which had lost pXO2 should be sensitive to MLS antibiotics. Four Cap^- $pXO2^-$ mutants isolated from such transductants were all found to be MLS^s. On the other hand, if a transductant carried Tn917 on the chromosome, as would be expected when tp50 was the donor, then spontaneous Cap^- mutants which had lost pXO2 should retain resistance to MLS antibiotics. Six Cap^- $pXO2^-$ mutants isolated from such transductants were all found to be MLS^r.

The above results suggest very strongly that in both tp49 and tp50 the insertion of Tn917 is responsible for the mutant phenotype. We plan to study these transposants further in an effort to understand how they differ genetically and physiologically from the wild-type parent strain.

Phenotype of transposant 4229 UM12 tp47. B. anthracis 4229 UM12 tp47 is a Cap^{c+} (Cap^{+} only in CO_2) mutant which does not grow when incubated in air without added CO_2. When cells are grown in medium containing sodium bicarbonate and incubated in 20% CO_2, the colonies are mucoid. When cells are incubated in air without added CO_2, there is a high frequency of revertants (or suppressed mutants) which grow in air. Such cells are probably more likely to be suppressed mutants than true revertants; they retain their resistance to MLS antibiotics and it seems unlikely that precise excision of Tn917 and insertion at another site could occur at such high frequency. Suppressed cells seem to be of two types. One type appears to "feed" the neighboring mutant cells (or consume or otherwise inactivate a substance which prevents their growth). The other type does not exhibit such activity. Under the microscope mutant cells appear as "healthy" normal cells and suppressed cells appear to be shorter, to have odd shapes, and to be frequently fragmented (lysed).

It has been difficult to obtain good plasmid extracts from this mutant. Cells for plasmid extraction could not be grown by the usual method in shaken 250-ml cotton-plugged flasks containing 25 ml of BHI broth with horse serum. When cultured in screw-capped flasks (to retain any CO_2 that was produced), the cells grew better but quite slowly and such cultures contained a significant proportion of suppressed cells, making it difficult to work with plasmid extracts from a pure strain. Plasmid extracts from such cells produced only faint bands in agarose gels.

Spores of 4229 UM12 tp47 were streaked on LPA plates with no sodium bicarbonate and incubated at 37°C in 20% CO_2. Under these conditions cells gave mostly rough colonies with only a few mucoid colonies. Rough and mucoid colonies were streaked on LPA plates and incubated at 37°C in air. Those colonies that were rough in CO_2 (on agar without bicarbonate) did not grow in air, and those that were mucoid in CO_2 produced colonies in air that resembled suppressed mutants. Taking advantage of this observation, we finally obtained reasonably good plasmid preparations by extracting cells grown on LPA plates with no sodium bicarbonate and incubated at 37°C in 20% CO_2 for 24 hours.

We have shown that both mutant and suppressed cells of tp47 contain pXO2 but because of the problems described above in obtaining good plasmid preparations from this transposant we have not been able to obtain definite proof that Tn917 is inserted into pXO2 rather than into the chromosome. We

believe that pXO2 is in the plasmid rather than the chromosome, and hopefully we will be able to confirm this now that we have been more successful in obtaining reasonably good plasmid preparations from the transposant.

Attempts to cure 4229 UM12 tp47 of pXO2 with novobiocin. In absence of confirming evidence that the Tn917 insertion in tp47 is in pXO2, or that the insertion itself is responsible for the mutant phenotype of tp47, we attempted to cure this strain of the plasmid by growing cells in the presence of novobiocin. However, we have been unsuccessful in finding any cured colonies.

Carbon dioxide-dependency of 4229 UM12 tp47 for growth. To determine whether tp47 actually requires CO_2 for growth or whether it grows in 20% CO_2 as a result of reduced oxygen tension, we tested the mutant for ability to grow on agar plates in different atmospheres: air, 5% CO_2, 20% CO_2, 80% N_2, and without O_2 in an anaerobic chamber. The media used in these experiments were as follows: LPA and minimal 1C for air, 80% N_2, and anaerobic chamber; $LPACO_3$ (LPA with sodium bicarbonate) and minimal $ICCO_3$ (minimal 1C with sodium bicarbonate) for CO_2. Cells grew as mutants (almost no growth) in air and 80% N_2, giving suppressed type of colonies only in air. They did not grow in the anaerobic chamber. In 5% and 20% CO_2 colonies were mucoid, but they grew more slowly in 5% CO_2 than in 20% CO_2.

To determine whether CO_2 is required only for initiation of growth of tp47 or whether it is required for sustained growth, cells were streaked on LPA plates which were incubated for 3 hours at 37°C in 5% or 20% CO_2 and then transferred to air at 37°C. The results were similar to those obtained when the plates were incubated in air with no previous CO_2 incubation. Thus, we conclude that tp47 has a definite requirement for CO_2, and although it grows in CO_2 without added bicarbonate, it requires bicarbonate for capsule formation.

The reason that 4229 UM12 tp47 does not grow in air remains unclear but it is probably not due to a nutritional requirement for CO_2. It seems more likely that the CO_2 requirement results from a defect in some regulatory gene.

Transfer of pXO2 from Cap^{a+} transposants to pXO2-cured strains by mating. To confirm that Tn917 insertion is responsible for the Cap^{a+} (Cap^{+} in air) phenotype observed in some transposants, the *B thuringensis* conjugation system was used to transfer pXO2 to strains previously cured of pXO2. The fertility plasmid pXO12 was introduced into 4229 UM12 tp58 Cap^{a+} using Weybridge A UM23-21(pXO12, pBC16) as a donor. MLS^{r} Tc^{r} transcipients of tp58

were selected on L agar containing inhibitory concentrations of erythromycin, lincomycin and tetracycline. Transconjugants were subsequently screened by phase microscopy for the presence of parasporal crystals and by plasmid profiles in agarose gel electrophoresis.

Samples of tp58 transcipients that had inherited both plasmids, pXO12 and pBC16, were then used as a donors in a second mating with B. anthracis Str^r, $pXO2^-$ strains as recipients. Transcipients were selected on L agar containing streptomycin, erythromycin and lincomycin. Capsule production in air was observed in all pXO2::Tn917-containing transcipients, resembling the phenotype from the donor strain.

Having now shown that capsulated cells can participate in conjugation, we will use this approach to transfer the pXO2::Tn917 derivative present in tp47 to other cells to test whether the phenotypic characteristics of tp47 are acquired by cells inheriting the plasmid. We can not transfer the plasmid of tp47 by transduction since the strain does not grow in air and it is capsulated when grown in CO_2. The transducing phage CP-51 does not infect capsulated cells.

Location of Tn917 in some Tn917-tagged pXO2 derivatives by restriction and DNA-DNA hybridization. Preliminary data obtained from EcoRI digestion of four pXO2::Tn917 derivatives showed that Tn917 had inserted into different sites in pXO2 as confirmed by DNA-DNA hybridization using ^{32}P-labelled Tn917 as the probe. Analysis showed that Tn917 had inserted into different regions on the four plasmids examined, thus accounting for the differences in the observed phenotypes. The following results were obtained: tp24, Cap^{a+}, insertion in a ca. 11.9-kb fragment; tp7, Cap^-, insertion in a ca. 6.2-kb fragment; tp49, Cap^{c+}, polypeptide overproducer, insertion in a ca. 8.8-kb fragment; tp24-17, a Cap^{c+} deletion-derivative (deletion greater than 70-kb), insertion in a ca. 7.9-kb fragment.

III. Characterization of the conjugative plasmid pLS20 of Bacillus subtilis

A minor part of our effort during the past year was spent on further characterization of the conjugative plasmid, pLS20, which encodes genes for plasmid transfer among strains of B. subtilis, B. cereus, B. anthracis, and B. thuringiensis. Included here is a review of the work on pLS20 that has been carried out since our first publication (7) on that subject.

We have identified a 10.8-kb BglII fragment of the plasmid that is responsible for the transfer proficiency of the host organism. Transposon insertions within this region generated mutant plasmids which showed alterations in the transfer phenotype, and deletions of this region rendered the host organism completely transfer-defective. Cloning of the 10.8-kb fragment into the gram-positive cloning vehicle, pBD64, rendered the host organism transfer proficient, thus confirming that the fragment is necessary and sufficient for conferring conjugal transfer ability on host cells.

Suppression of motility of B. subtilis cells harboring pLS20 is a second plasmid-encoded phenotype that can be attributed to the plasmid. This suppression of motility was observed with cells harboring pLS20 and transposon-tagged derivatives thereof, and reversion to wild-type motility was found in cells harboring deletion derivatives of the plasmids. Additionally, B. anthracis cells harboring pLS20 appeared much more dense in electron microscopic analysis than cells of the same strain not carrying pLS20, suggesting the presence of some sort of coating on the cells. Clewell et al. (5) reported that E. faecalis cells harboring the pheromone-responsive conjugative plasmid pPD1 produce a "fuzzy" coating thought to be an aggregation substance. Although pLS20 does not appear to encourage the formation of donor and recipient aggregates in the manner that pPD1 does, perhaps the coating on B. anthracis cells carrying pXO2 is related in some way to the mating event.

Generation of pLS20::Tn917 derivatives. Studies to localize the transfer-related sequences on pLS20 were initiated by the generation of pLS20 derivatives mutagenized by transposon insertion. The temperature-sensitive transposition selection vector pTV1 was employed to generate insertions of Tn917 in PSL1 UM3 (pLS20, pTV1, pBC16). Following induction of transposition and curing of pTV1 from these cells, the Cm^s MLS^r isolates were examined for the location of Tn917 within their genomes by BglII digestion of plasmid DNA isolated from cells. Tn917 is cleaved twice by BglII, thus yielding a characteristic 1.7-kb internal fragment.

Utilizing this approach we screened 150 independently isolated colonies for the location of the transposon. Tn917 exhibited a strong preference for insertion within plasmid DNA, as over 60% of the insertions obtained (97/150) were in pLS20 rather than chromosomal target sites. However, agarose gel electrophoresis revealed that among the 97 pLS20::Tn917 isolates examined by

BglII digestion, only 9 different restriction patterns were generated. No insertions in the 4.2-kb tetracycline resistance plasmid, pBC16, were observed. These pLS20::Tn917 derivatives were designated pXO505 to pXO513.

Transfer phenotypes of pLS20::Tn917 mutants. The B. subtilis transposants harboring one of the pLS20::Tn917 derivatives pXO505 through pXO513 and the nonconjugative, mobilizable plasmid pBC16 were tested for their ability to transfer the Tn917-tagged fertility plasmid or pBC16 to B. subtilis 168 UM47. Table 12 illustrates the representative transfer abilities of the mating phenotypes that resulted.

Transfer proficiencies of the donor cells were quite varied. Insertions of Tn917 in derivatives represented by pXO509 resulted in abolishment of or extreme reduction in transfer abilities, i.e., the transfer of the fertility plasmid and the transfer of pBC16 were either totally abolished or greatly reduced. These plasmids included pXO505, pXO507, pXO509, pXO510, and pXO513. Transposons insertions generating pXO506 and pXO508 abolished self-mobilization but functions required for mobilization of other plasmid DNA, e.g., pBC16, appeared to be intact as tetracycline-resistant transconjugants were obtained.

The insertion in pXO512 did not seem to affect the transfer abilities of the host organism; thus, the transposon had inserted within a region of pLS20 DNA that carries information unrelated to transfer. pXO511 represents still another phenotype. Inasmuch as the insertion of Tn917 in pXO511 did not appear to affect the ability of the plasmid to transfer itself if direct selection for MLS resistance was applied, the establishment of the transferred plasmid once in the recipient is probably affected. As shown in Table 12, the number of MLS^r transcipients was fairly high, 3.1×10^2. This indicates that the transposon insertion did not affect any locus specific for self-transfer. However, of the 22 tetracycline-resistant transcipients screened for co-acquisition of the fertility plasmid, none simultaneously inherited pXO511. This suggests that stable inheritance of the fertility plasmid was affected by the insertion of the transposon and, thus, pressure for plasmid maintenance had to be applied.

These data allowed the assignment of the pLS20::Tn917 derivatives to three distinct classes based on their transfer phenotypes. Class I plasmids, exemplified by pXO505, pXO507, pXO509, pXO510, and pXO513, are phenotypically transfer deficient, indicating insertional inactivation of a trans-acting

mating factor. Class II plasmids, exemplified by pXO506 and pXO508 are able to promote the mobilization of nonconjugative plasmids but cannot transfer themselves, suggesting that the insertions have interrupted a cis-acting region of the transfer DNA sequences. Class III plasmids include pXO511 and pXO512, in which the transfer potential of the plasmids are unaltered by transposon insertion.

To confirm that the aberrant transfer phenotypes were a direct result of the insertion of Tn*917* into transfer-associated DNA and were not a result of Tn*917*-induced plasmid instability, viable colony counts of PSL1 donor cells harboring pLS20::Tn*917* derivatives were done on nonselective agar and on plates containing erythromycin. As is evident in Table 13, the percentage of MLS^r cells in the populations did not correlate with transfer efficiencies of the particular donor cells. For example, populations harboring pXO509 or pXO512 showed comparable proportions of MLS^r cells, approximately 95%, but pXO509 was transfer deficient while pXO512 exhibited wild-type frequencies of plasmid transfer. In addition, the pLS20::Tn*917* derivatives pXO505 to pXO513 were introduced into a pBC16-containing rifampycin-resistant 168 strain either by mating or by protoplast transformation and then used as donors of MLS resistance and tetracycline resistance to Str^r 168 recipients to determine the effects of donor strain variation (PSL1 compared with 168) on the mating proficiencies. The numbers of transcipients obtained (data not shown) were comparable to the numbers of transcipients obtained from the matings shown in Table 12. Thus, the different transfer phenotypes exhibited by the donor cells were not the result of plasmid instability or differences in the cellular physiology of particular host cells. Rather the insertion of the transposon within pLS20 led directly to the aberrant transfer phenotypes of these pLS20::Tn*917* -containing donor cells.

Isolation of transfer-positive insertion mutants. Ninety three of the 97 pLS20::Tn*917* derivatives mentioned above contained insertions leading to defective transfer phenotypes. To obtain more insertions within pLS20 that did not affect a region of the plasmid encoding transfer functions, we used the procedure of Koehler and Thorne (7). A culture of *B. subtilis* PSL1 UM3(pLS20, pTV1, pBC16) that was induced for transposition of Tn*917* by growing in the presence of erythromycin and lincomycin at 48°C was used directly as inoculum for cells to be used as donors of MLS resistance to *B. subtilis* IG20 UM1 and 168 UM21 recipients in membrane matings. The procedure relied on the

observations that the transposition vector pTV1 could not be mobilized by pLS20 and no transposition of Tn*917* to pBC16 had been observed when the induction and curing experiments were performed in *B. subtilis* PSL1 UM3. Thus, any MLS^r transcipients obtained would presumably result from transfer of the transpson-tagged fertility plasmid. This procedure had previously allowed the isolation of four Tra^+ pLS20::Tn*917* derivatives, pXO501 to pXO504 (7).

Plasmid DNA was extracted from the MLS^r *B. subtilis* transcipients obtained and subjected to restriction endonuclease digestion with *Bgl*II to confirm that the transposon was located on the plasmid. The Tra^+ pLS20::Tn*917* derivatives, pXO501 to pXO504 were also analyzed by digestion with *Bgl*II. Results indicated that this method was successful in generating 7 different insertion derivatives: the three unique insertions in the previously isolated derivatives pXO501 to pXO504 (pXO503 and pXO504 appear to be identical as judged by restriction analysis) and four unique insertion derivatives isolated in this study, designated pXO514 to pXO517. Five of the seven derivatives contained insertions that were within the 27.0-kb *Bgl*II fragment of pLS20 (pXO501, pXO503, pXO514, pXO515, and pXO516), one derivative contained an insertion within the 14.4-kb *Bgl*II fragment (pXO502), and one contained an insertion in the 5.4-kb *Bgl*II fragment (pXO517). These results, in conjunction with the restriction data obtained from analysis of pXO505 to pXO513, provided evidence that the portions of the 27.0-kb, 14.4-kb, and 5.4-kb *Bgl*II fragments into which the transposon inserted do not encode functions necessary for conferring transfer proficiency upon a host organism.

The plasmids pXO501 to pXO504 and pXO514 to pXO517 were grouped into the Class III derivatives along with pXO511 and pXO512 described earlier. These plasmids conferred a Tra^+ phenotype upon the host organism identical to that conferred by the parental pLS20.

Isolation and characterization of pLS20::Tn*917* Deletions. The localization of transfer functions on pLS20 was aided by the study of deletion mutants. We observed that growth of some of the Tn*917*-tagged pLS20 derivatives, namely pXO503, pXO504, and pXO513, in the presence of selective levels of erythromycin resulted in the formation of deletions. Passage of the cells that harbored pXO503, pXO504, or pXO513 several times in LG broth supplemented with 1 μg of erythromycin per ml resulted in a deletion of ca. 16 kilobases of DNA, approximately one fourth of the pLS20 molecule. Once this DNA was lost the plasmids were stable and appeared to undergo no further

deletions. This phenomenon was demonstrated to occur when the plasmids were present either in B. subtilis or B. anthracis cells. The deletions occurred spontaneously at a high rate, and their formation was enhanced by growth in selective levels of erythromycin, suggesting that erythromycin was inducing the formation of deletions. Growth of strains carrying the other pLS20::Tn917 derivatives in selective levels of erythromycin did not yield detectable deletion derivatives, nor did wild-type pLS20 when passaged several times in LG broth. This, along with data mentioned below in regard to the specific and nonrandom nature of the deleted DNA and endpoints, suggests that the location of at least some of the genes required for plasmid maintenance are located proximal to the newly created junction of DNA fragments following deletion.

The B. subtilis and B. anthracis strains harboring the deletion derivatives were tested for the ability to promote conjugal transfer of erythromycin resistance and tetracycline resistance. The results showed that they were totally transfer-deficient. Neither pBC16 nor the deleted pLS20::Tn917 derivatives were transferred, suggesting that the pLS20 DNA sequences lost in the deletion derivatives were required to confer transfer proficiency upon the host organism.

Molecular characterization of the deletion derivatives. To confirm the location of the transposon on the deletion derivatives, they were digested with the restriction endonucleases AvaI, BglII, and StuI and the resulting fragments were tested for hybridization with ^{32}P-dGTP-labelled Tn917 DNA. Undeleted pX0503 was also treated in the same manner. The fragments showing homology to the probe DNA were as expected. The hybridization patterns indicated that only one copy of Tn917 was present in the deletion derivatives and the position of the transposon was located in the same region of the deleted plasmid as it was in the parental transposon-tagged plasmid.

The complement of restriction fragments generated by the above enzymes indicated that the size of the deletion derivative was ca. 54 kb, 5.2 kb of which was Tn917. The parental plasmid, pX0503, is 69.4-kb, including the 5.2-kb Tn917. Thus, the deletion derivative has lost approximately 16 kb of pLS20 DNA. The restriction patterns indicated that the region of pLS20 deleted in the derivative encompassed the 10.8-kb BglII fragment, as well as the 2.8 BglII fragment and a portion of the largest BglII fragment. The 14.4-kb, 6.5-kb, and 5.4-kb BglII fragments all appeared to be unaltered in the transfer defective deletion derivative. Restriction analysis of plasmids from random

single colony isolates from a culture of B. subtilis (pXO503) that had been passaged several times in broth containing erythromycin showed the majority of the cells (15 out of 16) contained plasmid DNA that exhibited identical restriction fragment patterns, providing strong evidence for specific deletions and nonrandom endpoints. These data, in conjunction with the insertion data presented earlier in which the insertion of Tn917 into the 10.8-kb BglII fragment led to aberrant transfer phenotypes, confirms that this region of pLS20 contains information necessary for conferring the transfer-positive phenotype upon the host cell.

Cloning of the transfer region of pLS20. Conclusive evidence that the 10.8-kb BglII fragment encodes all the information necessary for conferring transfer proficiency upon the host cell was provided by cloning the 10.8-kb BglII fragment into the 4.8-kb gram-positive insertional inactivation vector pBD64. The vector contains one cleavage site that is recognized by BglII within the gene encoding kanamycin resistance. pXO503 DNA isolated from B. subtilis 168 UM42 was restricted with BglII and the 10.8-kb fragment was purified by electroelution and ethanol precipitation. The fragment was ligated to pBD64 DNA that had been linearized by digestion with BglII. Protoplasts or competent cells of B. subtilis 168 UM47 or 168 UM48(pBC16) were transformed with the ligation mixture and Cm^r transformants were selected. Transformants were then picked to kanamycin plates to identify colonies carrying Cm^r Km^s recombinant molecules.

Those cells harboring recombinant plasmids were tested as donors of chloramphenicol resistance to B. subtilis 168 UM21. Transcipients were obtained from those membrane matings in which the donor cell contained a pBD64::10.8-kb plasmid derivative. Confirmation that the 10.8-kb BglII fragment was, in fact, cloned into pBD64 was obtained by DNA-DNA hybridization utilizing ^{32}P-labelled 10.8-kb BglII fragment of pLS20.

The Tra^+ recombinant plasmid is designated pXO41. B. subtilis 168 UM63(pXO41) was employed as a donor of chloramphenicol resistance to B. subtilis IG20 UM10(pLS19). One of the resulting Cm^r transcipients, IG20 UM11(pXO41, pLS19), was then used as a donor to test for the presence of necessary trans-acting mating factors on the recombinant plasmid. T. Koehler had previously shown in my laboratory that pLS20 is capable of mobilizing the cryptic plasmid pLS19; thus, it was a suitable choice for testing the capabilities of pXO41 to mobilize other plasmids. Plasmid profiles of the

resulting Cm^r transcipients revealed that ca. 25% of them acquired pLS19 along with the recombinant plasmid pXO41. Thus, the 10.8-kb BglII fragment of pLS20 is necessary and sufficient for conferring transfer proficiency upon its host.

Physical map of pLS20. Restriction analysis of pLS20, the pLS20::Tn917 derivatives, and the Tra^- deletion derivatives has allowed the construction of a partial physical and genetic map of pLS20. The physical organization of the 64.2-kb plasmid is shown in Fig. 1.

Electron microscopic studies of pLS20-containing host cells. Conjugative mobilization of plasmids sometimes involves the production of transfer-specific macromolecules such as the pili encoded by the F plasmid or the clumping inducing agents specified by the streptococcal fertility plasmids in the pheromone-directed systems. To determine whether pLS20 encoded any type of macromolecular surface component, electron microscopy was employed to compare the surface of cells harboring pLS20 to the surface of cells which did not contain the plasmid. Cells of both B. subtilis and B. anthracis in log phase growth were stained with uranyl acetate and examined by scanning electron microscopy.

No pili-type structures were apparent. However, B. subtilis cells containing pLS20 did not have flagella, in contrast to B. subtilis cells of the same strain but not containing pLS20 which were characteristically peritrichously flagellated. B. anthracis cells are nonmotile and do not have flagella. However, B. anthracis cells carrying pLS20 appeared to be more dense than cells of the same strain not carrying pLS20, suggesting the presence of a new component on the cell surface.

A variety of B. subtilis strains harboring pLS20 were examined for their ability to produce flagella. This was accomplished by screening cells for their motility phenotype in 0.4% agar plates. The results were the same for all strains tested; the B. subtilis cells harboring pLS20 were nonmotile whereas their plasmid-free parents were highly motile.

After several days of incubation some outgrowth appeared from the pLS20-containing colonies on 0.4% agar plates. When this outgrowth was inoculated into the center of fresh soft agar plates, the cells were found to be motile. Examination of the DNA content of cells from the center of the colony and cells from the edge of the motility plate revealed that those cells which had migrated toward the edge of the plate had lost pLS20. This provided additional evidence that pLS20 was responsible for the suppression of motility

of its host cell, as curing of pLS20 from the cells was concomitant with the return of motility. Thus, these observations indicate that the presence of pLS20 suppresses the production of flagella rendering the host cell nonmotile.

Motility of cells containing pLS20::Tn917 plasmids. All pLS20-containing strains of B. subtilis tested were nonmotile. The pLS20::Tn917-containing strains were also screened in a similar manner to determine whether Tn917 had inserted into the region of pLS20 responsible for suppression of motility. As with the parental plasmid pLS20, all the Tn917-tagged derivatives also suppressed motility. Thus, none of the Tn917 insertions occurred within the region of pLS20 involved in suppression of motility. After several days of incubation of motility agar plates, however, some motile outgrowth was observed from cells containing pXO503 and pXO504. Plasmid analysis indicated that nonmotile cells taken from the point of inoculation contained the parental pLS20::Tn917 plasmid, whereas motile cells from the outgrowth either contained a deleted from of the plasmid or had been cured of the plasmid. This indicated that the region of pLS20 responsible for suppression of motility appears to be within the ca. 16-kb of pLS20 DNA that is lost in the deletion derivatives. When B. subtilis 168 UM63 harboring the Tra^+ recombinant plasmid, pXO41, was inoculated into the center of a soft agar plate, a colony of nonmotile cells formed at the point of inoculation. However, the parent strain, B. subtilis 168 UM47 from which 168 UM63 was derived, was motile. Thus, the region of pLS20 that suppresses motility is probably within the 10.8-kb BglII fragment and its function may be related to transfer proficiency.

TABLE 1. Bacterial strains, plasmids, and bacteriophages

Strain or plasmid	Relevant characteristics[a] and plasmids	Origin or Reference[b]
B. anthracis		
Weybridge	Avirulent, Tox^+ Cap^- pXO1	14
Weybridge UM44	Ind^- Tox^+ Cap^- pXO1	UV of Weybridge
Weybridge UM44-1	Ind^- Tox^+ Str^r pXO1	UV of UM44
Weybridge UM44-1C9	Ind^- Tox^- Str^r $(pXO1)^-$	Curing of UM44-1
Weybridge UM44-1C9 td13 and td14	Ind^- Str^r pXO1.1::Tn*917*Δ1	This study
Weybridge UM44-1C9 tr1 to tr4	Ind^- Str^r MLS^r pXO1.1::Tn*917*	This study
Weybridge A	Colony variant of Weybridge, Tox^+ pXO1.1	14
Weybridge A UM23	Ura^- Tox^+ pXO1.1	UV of Weybridge A
Weybridge A UM23-1	Ura^- Tox^+ pXO1.1 Str^r	UV of UM23
Weybridge A UM23-2 (same as A UM23C1)	Ura^- Tox^- $(pXO1.1)^-$	Curing of A UM23
Weybridge A UM23-3 (same as A UM23C1-1)	Ura^- Tox^- Str^r $(pXO1.1)^-$	UV of A UM23-2
Weybridge A UM23 tp2A	Ura^- MLS^r pXO1.1::Tn*917*	This study
Weybridge A UM23 tp18	Ura^- MLS^r pXO1.1::Tn*917*	This study
Weybridge A UM23 tp21	Ura^- MLS^r pXO1.1::Tn*917*γ1	This study
Weybridge A UM23 tp26	Ura^- MLS^r pXO1.1::Tn*917*	This study
Weybridge A UM23 tp27	Ura^- MLS^r pXO1.1::Tn*917*	This study
Weybridge A UM23 tp28	Ura^- MLS^r pXO1.1::Tn*917*	This study
Weybridge A UM23 tp29	Ura^- MLS^r pXO1.1::Tn*917*	This study
Weybridge A UM23 tp32	Ura^- MLS^r pXO1.1::Tn*917*	This study

Continued next page

Table 1 (continued)

Weybridge A UM23 tp38	Ura$^-$ MLS^r pXO1.1::Tn*917*	This study
Weybridge A UM23 tp43 to tp76	Ura$^-$ MLS^r pXO1.1::Tn*917*	This study
Weybridge A UM23 tp62 tr2	Ura$^-$ MLS^r Tc^r pXO1.1::Tn*917*, pXO12::Tn*917*, pBC16	This study
Weybridge A UM23 tp62 td1 to td3	Ura$^-$ MLS^r Tc^r Cm^r pXO1.1::Tn*917*, pTV53	This study
Weybridge A UM23 tp62 td2C1 to C4	Ura$^-$ MLS^r Cm^r pXO1.1::Tn*917*, (pTV53)$^-$	This study
Weybridge A UM23-1 etf3	Ura$^-$ Str^r MLS^r Tc^r pXO1.1, pTV53	This study
Weybridge A UM23-1 etf5 and etf6	Ura$^-$ MLS^r Cm^r Tc^r pXO1.1, pLTV3	This study
Weybridge A UM23-1 ctf1	Ura$^-$ MLS^r Cm^r Tc^r pXO1.1, pLTV3	This study
Weybridge A UM23C1	Ura$^-$ (pXO1.1)$^-$	C.B. Thorne
Weybridge A UM23C1 tds1	Ura$^-$ MLS^r pXO1.1::Tn*917*Δ1	This study
Weybridge A UM23C1 tds1C1 and C2	Ura$^-$ MLS^r (pXO1.1::Tn*917*Δ1)$^-$	This study
Weybridge A UM23C1-2	Ura$^-$ Rif^r (pXO1.1)$^-$	C.B. Thorne
Weybridge A UM23C1-2 tr1 to tr4	Ura$^-$ Rif^r MLS^r pXO1.1::Tn*917*	This study
4229 (Pasteur)	Cap$^+$ Tox$^-$ pXO2	6
4229 UM12	Cap^{c+} Tox$^-$ Nal^r pXO2	C. B. Thorne
6602 (Pasteur)	Cap$^+$ Tox$^-$ pXO2	6
B. cereus		
569	Wild type	NRRL
569 UM20-1	Ant$^-$ Str^r	C. B. Thorne
B. subtilis		
PA1	pPA101	USAMRIID
PY332	MLS^r Tc^r pTV53	P. Youngman
PY1178	MLS^r Cm^r Tc^r pLTV3	P. Youngman

Continued next page

Table 1 (continued)

PY143	trpC2 Cm^r MLS^r pTV1	P. Youngman
168	trpC2	C. B. Thorne
168 UM21	leu-8 metB5 str-2	C. B. Thorne
168 UM42	leu-8 metB5 str-2 MLS^r Tra^+ pXO503	T. Koehler
168 UM47	trpC2 Rif^r	T. Koehler
168 UM48	trpC2 str-1 Tc^r pBC16	$tfn^{(f)}$ of 168 UM1
PSL1	r^- m^- Arg^- Thr^- Leu^- Rec^-	BGSC strain 1A510
PSL1 UM1	r^- m^- Arg^- Thr^- Leu^- Str^r Rec^-	Spontaneous from PSL1
PSL1 UM2	MLS^r Cm^r	pTV1 C.B. Thorne
PSL1 UM3	pLS20, pTV1, pBC16	T. Koehler
IG-20	r^- m^- trpC2	GSC strain 1A436
IG-20 UM1	r^- m^- trpC2 Rif^r	UV of IG-20
IG20 UM10	trpC2 r^- m^- MLS^r Rif^r Tc^r pLS19, pBC16	T. Koehler
Bacillus subtilis (natto)		
3335	Pga^+ Tra^+ pLS19, pLS20	T. Hara
3335 UM4	Pga^- Tra^+ pLS20, $(pLS19)^-$	T. Koehler
3335 UM25	Pga^- Tra^- $(pLS20)^-$, $(pLS19)^-$	T. Koehler
3335 UM27	Pga^- Tra^+ Tc^r pLS20, pBC16	T. Koehler
E. coli		
HD101	pSE42	USAMRIID
JM103	pLF7	USAMRIID
Bacteriophages		
CP-51	Generalized transducing phage	C. B. Thorne
CP-51ts45	Temperature-sensitive mutant of CP-51	C. B. Thorne

Continued next page

Table 1 (continued)

CP-54	Generalized transducing phage, more lytic than CP-51	C. B. Thorne
Plasmids		
pBC16	Tc^r	3
pBD64	Cm^r Km^r, hybrid of pUB110 and pC194	P. Lovett
pLF7	Carries LF structural gene	USAMRIID
pLS19	Pga^+	T. Hara
pLS20	Tra^+ plasmid of B. subtilis (natto)	T. Hara
pTV1ts	MLS^r Cm^r, temperature-sensitive transposition selection vector	Youngman
pTV53	Carries Tn917 and promotorless cat and lac genes	Youngman
pLTV3	Temperature-sensitive transposition selection vector	Youngman
pPA101	Carries PA structural gene	USAMRIID
pSE42	Carries EF structural gene	USAMRIID
pXO1	Toxin plasmid from B. anthracis Weybridge	9, 14
pXO1.1	Toxin plasmid from Weybridge A	C. B. Thorne
pXO2	Encodes synthesis of B. anthracis capsule	6
pXO12	Tra^+ Cry^+ from B. thuringiensis 4042A	2
pXO41	Tra^+ Cm^r Km^s pBD64::10.8-kb	This study
pXO501 to pXO504	pLS20::Tn917, MLS^r $Tra^{+(c)}$	T. Koehler
pXO505	pLS20::Tn917, MLS^r $Tra^{-(c)}$	This study
pXO506	pLS20::Tn917, MLS^r $Tra^{+(d)}$	This study

Continued next page

Table 1 (continued)

pXO507	pLS20::Tn*917*, MLS^r $Tra^{-(c)}$	This study
pXO508	pLS20::Tn*917*, MLS^r $Tra^{+(d)}$	This study
pXO509	pLS20::Tn*917*, MLS^r $Tra^{-(c)}$	This study
pXO510	pLS20::Tn*917*, MLS^r $Tra^{-(c)}$	This study
pXO511	pLS20::Tn*917*, MLS^r $Tra^{+(e)}$	This study
pXO512	pLS20::Tn*917*, MLS^r $Tra^{+(e)}$	This study
pXO513	pLS20::Tn*917*, MLS^r $Tra^{-(c)}$	This study
pXO514 to pXO517	pLS20::Tn*917*, MLS^r $Tra^{+(d)}$	This study

[a] Abbreviations: Ade, adenine; Ant, anthranilic acid; Ind, indole; Trp, tryptophan; Ura, uracil; MLS^r, Tn*917*-encoded macrolide, lincosamide, and streptogramin B resistance; Rif^r, rifampicin resistant, Str^r, streptomycin resistant; Tc^r, pBC16 encoded tetracycline resistance; Cap, synthesis of polyglutamate capsule; Tox, synthesis of anthrax toxin. Cm^r, pTV1- or pBD64-encoded chloramphenicol resistance; Cry, synthesis of parasporal crystal; Pga, synthesis of polyglutamic acid; Tra, mediation of plasmid transfer by mating; r^- m^-, restriction and modification deficient; Arg, arginine; Thr, threonine; Leu, leucine; Met, methionine.

[b] BGSC, *Bacillus* Genetics Stock Center, Columbus, Ohio; NRRL, Northern Regional Research Laboratory, Department of Agriculture, Peoria, IL; USAMRIID, US Army Medical Research Institute of Infectious Diseases; UV, mutagenesis by UV light (13).

[c] These pLS20::Tn*917* derivatives are designated as Class I plasmids and are Tra^-. They are unable to promote the transfer of either themselves or pBC16.

[d] These pLS20::Tn*917* derivatives are designated as Class II plasmids. They are incapable of self-mobilization but can promote the mobilization of pBC16.

[e] These pLS20::Tn*917* derivatives are designated Class III plasmids. The transfer phenotype of these plasmids is essentially equal to that of pLS20 in that mobilization of the fertility factor and pBC16 are not affected by the insertion of the transposon.

[f] tfn, transformation.

TABLE 2. Location of the toxin structural genes on pXO1.1

Probe[a]	Toxin gene[b]	Hybridization to fragments (kb) from pXO1.1 digested with		
		BamHI	PstI	EcoRI
pPA101	pag	6.0	18.1, 6.0	6.2, 5.6
pLF7	lef	34.8	6.6, 6.0	5.8, 5.6, 0.7
pSE42	cya	7.4	18.1, 5.5	8.0, 3.1, 1.1

[a] The probes were radiolabelled with ^{32}P-dGTP by nick translation.

[b] pag, PA structural gene; lef, LF structural gene; cya, EF structural gene.

TABLE 3. Phenotypic characterization of B. anthracis strains containing pXO1.1::Tn917 derivatives

Strain[a]	Phenotypic characteristics		
	Halo assay[b]	Sporulation[c] at 37°C	Sensitivity to CP-51[d]
UM23 (pXO1.1$^+$)	+	Osp	-
UM23C1 (pXO1.1$^-$)	-	Spo$^+$	+
UM23 tp43	+	Osp	-
UM23 tp44	-	Osp	-
UM23 tp45	+	Osp	-
UM23 tp46	-	Osp	-
UM23 tp47	-	Osp<Spo$^+$	-
UM23 tp48	-	Osp	-
UM23 tp49	+	Spo$^+$	+[e]
UM23 tp51	-	Osp	-
UM23 tp52	-	Asp	-
UM23 tp54	-	Asp	-
UM23 tp60	-	Osp	-
UM23 tp62	+[g]	Asp	-
UM23 tp63	-	Osp	-
UM23 tp64	+	Osp<Spo$^+$	-
UM23 tp65	+	Osp	+[f]
UM23 tp66	+	Osp	-
UM23 tp67	-	Asp	-
UM23 tp69	-	Osp	-
UM23 tp71	-	Spo$^+$	+[e]
UM23 tp75	+	Osp	-
UM23 tp76	+	Osp	-

[a] tp, transposant.
[b] +, strains formed halo (PA$^+$); -, PA$^-$.
[c] Extent of sporulation was determined by phase-contrast microscopy. Asp, asporogenous, (further analysis is necessary to confirm this phenotype); Osp, oligosporogenous; Osp<Spo$^+$, extent of sporulation greater than Osp, but less than Spo$^+$; Spo$^+$, extensive sporulation.
[d] +, plaque formation; -, no detectable plaques.
[e] Very small plaques compared to those on pXO1.1$^-$ strain.
[f] Plaques similar in size to those on pXO1.1$^-$ strain but more turbid.
[g] PA produced in the presence or absence of added bicarbonate and CO_2.

TABLE 4. PA production by Weybridge A UM23 and UM23 tp62 grown statically in CA broth[a]

Strain	CA broth[c]	PA assay[b] of samples taken at		
		15 h	20 h	25 h
UM23 tp62	pH 7.0	1	2	2
UM23 tp62	pH 7.5	2	8	16
UM23 tp62	pH 8.0	2	8	16
UM23 tp62	pH 8.5	<1	4	16
UM23 tp62	plus $NaHCO_3$	1	8	32
UM23	pH 7.0	<1	<1	<1
UM23	pH 7.5	1	1	1
UM23	pH 8.0	1	2	2
UM23	pH 8.5	2	4	8
UM23	plus $NaHCO_3$	<1	4	16

[a] Cultures were grown statically in 100 ml of the designated CA broth in 250-ml cotton-plugged flasks at 37°C.

[b] PA titers are expressed as the reciprocal of the highest dilution that produced a detectable line of precipitation.

[c] The CA broth contained no bicarbonate except where indicated.

TABLE 5. PA production by Weybridge A UM23 and UM23 tp62 in shaken flasks of CA broth[a]

Strain	CA broth[c]	PA assay[b] of samples taken at				
		5 h	10 h	15 h	25 h	30 h
UM23 tp62	pH 6.5	<1	<1	<1	<1	<1
UM23 tp62	pH 7.0	<1	4	4	2[d]	<1
UM23 tp62	pH 7.5	<1	8	32	2[d]	<1
UM23 tp62	pH 8.0	<1	<1	32	2[d]	<1
UM23 tp62	pH 8.5	<1	<1	8	16	<1
UM23 tp62	Plus $NaHCO_3$	NG[e]	NG	NG	NG	NG
UM23	pH 6.5	<1	<1	<1	<1	<1
UM23	pH 7.0	<1	2	<1	<1	<1
UM23	pH 7.5	<1	4	1[f]	<1	<1
UM23	pH 8.0	<1	2	1[f]	<1	<1
UM23	pH 8.5	<1	<1	8	<1	<1
UM23	Plus $NaHCO_3$	<1	<1	<1	<1	<1

[a] Cultures were grown at 37°C in 100 ml of the designated CA broth in 250-ml cotton-plugged flasks shaken at 100 rpm.

[b] PA titers are expressed as the reciprocal of the highest dilution which produced a visible line of precipitation.

[c] The CA broth contained no bicarbonate except where indicated.

[d] Precipitin band was formed at a position near the antiserum well unlike the precipitin band for PA which was formed closer to the sample well. This may represent a degraded form of PA.

[e] NG, no growth.

[f] No line of identity was observed with the PA band.

TABLE 6. Altered toxin phenotypes of some Weybridge A UM23 strains containing pXO1.1::Tn917 derivatives

Strain	Altered phenotypes[a]
UM23 tp62	PA^{a+}, LF^{a+}
UM23 tp18	PA^{-}
UM23 tp26	PA^{-}
UM23 tp29	$PA^{+/-(b)}$, $EF^{-(b)}$, $LF^{+/-(b)}$
UM23 tp32	$PA^{-(b)}$, $EF^{+(b)}$, $LF^{+/-(b)}$
UM23 tp38	PA^{-}
UM23 tp27	No alterations[b]

(a) PA^{a+} and LF^{a+}, the designated phenotypes for mutants that produce PA and LF, respectively, in medium containing no added bicarbonate and incubated in air without added CO2. The phenotype for LF^{a+} was determined by T. Koehler in J. Collier's laboratory at Harvard University.

(b) The phenotypes for the toxin components (PA, EF, and LF) were determined by Dr. S. Leppla at USAMRIID.

TABLE 7. BamHI restriction profiles of pXO1.1 and pXO1.1::Tn917 derivatives

BamHI fragments (kb) from[a]							
pXO1.1	tp62[b]	tp18[b]	tp26[b]	tp29[b]	tp32[b]	tp38[b]	tp27[b]
	38.7						
38.7	38.7	38.7	38.7	38.7	38.7	38.7	38.7
34.8		34.8	34.8	34.8	34.8	34.8	34.8
26.2	26.2	26.2	26.2	26.2	26.2	26.2	26.2
19.9	19.9	19.9	19.9	19.9	19.9	19.9	19.9
		19.5	**19.5**	**19.5**[c]	**19.5**[c]	**19.5**	**19.5**[c]
14.6	14.6	14.6	14.6	14.6	14.6	14.6	14.6
13.9	13.9						
7.7	7.7	7.7	7.7	7.7	7.7	7.7	7.7
7.4	7.4	7.4	7.4	7.4	7.4	7.4	7.4
7.1	7.1	7.1	7.1	7.1	7.1	7.1	7.1
6.6	6.6	6.6	6.6	6.6	6.6	6.6	6.6
6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0
3.9	3.9	3.9	3.9	3.9	3.9	3.9	3.9

[a] Boldface numbers represent new fragments not present in the profile of wild-type pXO1.1.

[b] The BamHI restriction profile was determined for pXO1.1::Tn917 derivatives isolated from these transposants.

[c] These new BamHI fragments contained Tn917 as determined by DNA-DNA hybridizations using ^{32}P-labelled Tn917 as the probe.

TABLE 8. BamHI and PstI restriction profiles of pXO1.1::Tn917 deletion derivatives from Weybridge A UM23 tp49 and tp71

BamHI fragments (kb) from[a]			PstI fragments (kb) from[a]		
pXO1.1	tp49[b]	tp71[b]	pXO1.1	tp49[b]	tp71[b]
	ca 40.0		18.7		
38.7			18.1		
34.8		34.8	16.2		
26.2					**ca. 16.0**
19.9				**ca. 12.9**	
14.6			11.6		
		ca. 13.9	10.7		
13.9			10.7		
7.7			10.1		
7.4			9.3		
7.1			9.3	9.3	9.3
6.6			6.6		
6.0			6.6	6.6	6.6
3.9			6.3		6.3
			6.0	6.0	
			5.7		
			5.5		
			5.2	5.2	5.2
			4.7		
			4.1		
			3.4		
			2.8		2.8
			2.1		
			1.7		
			1.3		
			1.1		
			1.0		
			0.8		
			0.7		
			0.5		
			0.4		
			0.3		

[a] Boldface numbers represent fragments not typically observed in BamHI or PstI digests of wild-type pXO1.1. These fragments were shown to contain Tn917 as determined by DNA-DNA hybridizations using ^{32}P-labelled pTV1 as the probe.

[b] The BamHI or PstI restriction profile was determined for pXO1.1::Tn917 deletion derivatives isolated from tp49 or tp71.

TABLE 9. Location of Tn917 and toxin structural genes on pXO1.1::Tn917 deletion derivatives from Weybridge A UM23 tp49 and tp71

Probe[a]	Hybridization to BamHI fragments (kb) from			Hybridization to PstI fragments (kb) from		
	pXO1.1	tp49[b]	tp71[b]	pXO1.1	tp49[b]	tp71[b]
Tn917	-[c]	40.0	13.9	-	12.9	16.0
pPA101	6.0	40.0	-	18.1, 6.0	12.9, 6.0	-
pLF7	34.8	40.0	34.8	6.6, 6.0	6.6, 6.0	16.0, 6.6
pSE42	7.4	-	-	18.1, 5.5	-	-

[a] The probes were radiolabelled with ^{32}P-dGTP by nick translation. Tn917 was isolated from pTV1; pPA101, pLF7, and pSE42 contain the PA, LF, and EF structural genes, respectively.

[b] BamHI or PstI fragments were generated from digestion of pXO1.1::Tn917 deletion derivatives isolated from tp49 and tp71.

[c] -, no hybridization detected.

TABLE 10. Phenotypic characteristics of Weybridge A UM23C1 tds1(pXO1.1::Tn*917*Δ1)[a] and its derivatives

Strain	Phenotypic characteristics	
	Sporulation[b] at 37°C	Sensitivity to CP-51[c]
A UM23 (pXO1.1$^+$)	Osp	-
A UM23C1 (pXO1.1$^-$)	Spo$^+$	+
UM44-1 (pXO1$^+$)	Spo$^+$	+
UM44-1C9 (pXO1$^-$)	Spo$^+$	+
A UM23C1 tds1(pXO1.1::Tn*917*Δ1)	Osp	-
A UM23C1 tds1C1(pXO1.1::Tn*917*Δ1)$^-$	Spo$^+$	+
A UM23C1 tds1C2(pXO1.1::Tn*917*Δ1)$^-$	Spo$^+$	+
UM44-1C9 td13(pXO1.1::Tn*917*Δ1)	Osp	-
UM44-1C9 td14(pXO1.1::Tn*917*Δ1)	Osp	-

[a] pXO1.1::Tn*917*Δ1 is a deletion derivative of pXO1::Tn*917* obtained by CP-51-mediated transduction of pXO1.1::Tn*917* from Weybridge A UM23 tp2A to UM23C1(pXO1)$^-$.

[b] Osp, oligosporogenous; Spo$^+$, extensive sporulation.

[c] +, plaque formation; -, no detectable plaques.

TABLE 11. Phenotypes of B. anthracis 4229 UM12 transposants

Transposant	Phenotype[a]	Tn917 insertion	Transposant	Phenotype[a]	Tn917 insertion
tp1	Cap^{c+}	pXO2	tp32	Cap^{c+}	pXO2
tp3	Cap^{+c}	pXO2	tp33	Cap^{c+}	pXO2
tp4	Cap^{c+}	pXO2	tp34	Cap^{c+}	pXO2
tp5	Cap^{c+}	pXO2	tp36	Cap^{-}	pXO2
tp6	Cap^{-}	pXO2 (deletion)	tp38	Cap^{-}	pXO2 (deletion)
tp7	Cap^{-}	pXO2	tp40	Cap^{-}	pXO2
tp10	Cap^{c+}	pXO2	tp45	Cap^{c+} NG air	?[b]
tp11	Cap^{c+}	pXO2	tp46	Cap^{c+} NG air	?
tp13	Cap^{c+}	pXO2	tp47	Cap^{c+} NG air	?
tp14	Cap^{c+}	pXO2	tp48	Cap^{c+} NG air	?
tp15	Cap^{c+}	pXO2	tp49	Cap^{c+} Op	pXO2
tp16	Cap^{c+}	pXO2	tp50	Cap^{c+} Op	Chromosome
tp17	Cap^{c+}	pXO2	tp51	Cap^{c+} Op	Chromosome
tp18	Cap^{-}	pXO2	tp53	Cap^{c+} Op	Chromosome
tp20	Cap^{-}	pXO2	tp54	Cap^{c+}	pXO2
tp21	Cap^{-}	pXO2	tp55	Cap^{c+}	Chromosome
tp22	Cap^{-}	pXO2	tp58	Cap^{a+} NG CO_2	pXO2
tp23	Cap^{-}	pXO2	tp59	Cap^{a+}	pXO2
tp24	Cap^{a+}	pXO2	tp60	Cap^{a+}	pXO2
tp24-17	Cap^{c+}	pXO2 (deletion)	tp62	Cap^{a+}	pXO2
tp25	Cap^{a+}	pXO2	tp63	Cap^{a+}	pXO2
tp30	Cap^{c+}	pXO2	tp64	Cap^{a+}	pXO2
			tp65	Cap^{a+}	pXO2

[a] Cap^{-}, does not produce capsules in presence or absence of bicarbonate and CO_2; Cap^{c+}, produces capsules only in presence of bicarbonate and CO_2 ; Cap^{a+}, produces capsules in air, i.e., in the absence of added bicarbonate and CO_2; NG air, no growth in air without added CO_2; NG CO_2; no growth in 20% CO_2; Op, appears to produce more glutamyl polypeptide than wild type.

[b] ?, location of Tn917 not determined conclusively.

TABLE 12. Donor activities of representative B. subtilis PSL1 strains harboring pLS20::Tn917 derivatives[a]

Plasmids of donor strain	MLS^r transcipients			Tc^r transcipients		
	No./ml	Frequency (no./donor cell)	% Cotransfer[b]	No./ml	Frequency (no./donor cell)	% Cotransfer[c]
pXO508, pBC16	0	0	NA[d]	6.1×10^2	3.4×10^{-6}	0. (0/34)
pXO509, pBC16	2.0×10^1	6.0×10^{-8}	50. (1/2)	1.0×10^1	3.2×10^{-8}	100. (1/1)
pXO511, pBC16	3.1×10^2	1.9×10^{-5}	77. (17/22)	6.9×10^3	4.3×10^{-4}	0. (0/22)
pXO512, pBC16	4.1×10^2	2.0×10^{-5}	65. (17/26)	2.3×10^4	1.1×10^{-3}	55. (12/22)
pXO513, pBC16	0	0	NA	0	0	NA
pLS20, pBC16	NA	NA	NA	8.0×10^3	3.8×10^{-4}	60. (12/20)

[a] The recipient was B. subtilis 168 UM47. The numbers of transcipients are the averages obtained from at least three membrane mating experiments.

[b] Percentage of MLS^r transcipients which also acquired pBC16 as indicated by results of plasmid extraction and/or tests for Tc^r. Numbers in parentheses represent the number of Tc^r transcipients over the number of MLS^r transcipients tested.

[c] Percentage of Tc^r transcipients which also acquired pLS20 or pLS20::Tn917 as indicated by results of plasmid extraction and/or tests for MLS^r resistance. Numbers in parentheses represent the number of MLS^r transcipients over the number of Tc^r transcipients tested.

[d] NA, not applicable.

TABLE 13. Viable colony counts of B. subtilis donor cells harboring pLS20::Tn917 derivatives[a]

Plasmid	CFU/ml on L agar	CFU/ml on LEL agar	% MLS^r
pXO508	3.5×10^8	3.3×10^8	94%
pXO509	6.2×10^8	5.7×10^8	92%
pXO511	3.1×10^7	3.1×10^7	100%
pXO512	4.2×10^7	4.0×10^7	95%
pXO513	3.1×10^7	3.0×10^7	97%

[a] The number of colony forming units per ml was determined by diluting the log-phase culture in 1% peptone and plating the appropriate dilutions on L agar and L agar supplemented with 1 µg of erythromycin and 25 µg of lincomycin per ml. The percentage of cells retaining the pLS20::Tn917 derivative was determined by dividing the number of MLS^r cells by the total number of cells in the population.

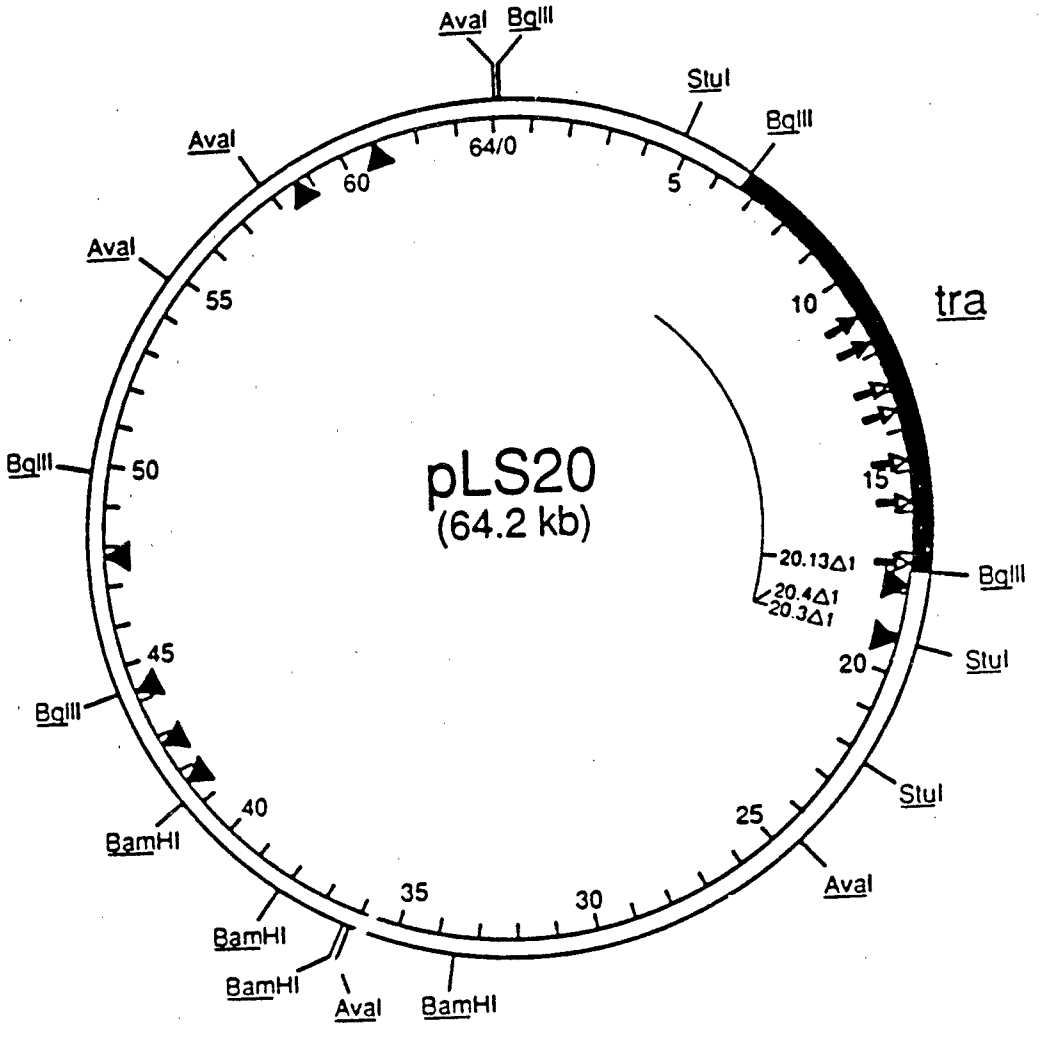

FIG. 1. Physical and functional map of pLS20. The map was constructed with an AvaI site taken as the reference point. Sequences lost from the deletion derivatives are shown by the innermost arc and are assigned the numbers of the plasmid designations; e.g., 20.3Δ1 represents the DNA lost from pXO503Δ1. The deletions encompassed ca. 16 kb of DNA and rendered the host cell transfer-deficient. The tra region is represented by the filled in area. Arrows are positioned at sites of Tn917 insertions that are contained within the 10.8-kb BglII fragment which contains the tra region. All insertions within the tra region led to aberrant transfer phenotypes. Insertions represented on the map as dark arrows in the area of map coordinates 11 and 12 allowed mobilization of pBC16 but led to abolishment of self-transfer. All other insertions within the 10.8-kb BglII fragment, denoted by empty arrows, led to a Tra^- phenotype. Insertions denoted by solid triangles had no effect on transfer phenotype.

PUBLICATIONS

The following paper was published during this reporting period:

Heemskerk, D. D., and C. B. Thorne. 1990. Genetic exchange and transposon mutagenesis in Bacillus anthracis. Salisbury Medical Bulletin, Number 68, Special Supplement 63-67.

The following Ph. D. dissertation was written on research carried out under this contract:

Jaworski, Deborah D. Physical and genetic analysis of the Bacillus subtilis (natto) fertility plasmid pLS20. Ph. D. Dissertation. University of Massachusetts, Amherst. May 1990.

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FINAL REPORT

I. Summary of Research

This is a summary of research on Bacillus anthracis carried out under contract DAMD17-85-C-5212 during the five-year period August 1, 1985-July 31, 1990. Pertinent references to specific Annual Reports or publications in the scientific literature are indicated where appropriate.

The primary objective of the research was to gain information and to develop genetic systems that will contribute to the development of an improved vaccine for anthrax. The Weybridge (Sterne) strain of B. anthracis was used primarily. This is an avirulent strain which is capable of producing toxin but incapaole of producing capsules and has been used widely as a live spore vaccine in domestic animals. Two other strains of B. anthracis that were used frequently in the research are B. anthracis ATCC 6602 and 4229. These strains, also avirulent or of low virulence, are capable of producing capsules but incapable of producing toxin.

Transformation of B. anthracis and Bacillus cereus

A number of reports have appeared in the literature on methods for transforming B. anthracis, B. thuringiensis, or B. cereus with plasmid DNA. However, until fairly recently we were unsuccessful in transforming B. cereus and B. anthracis by most, if not all, of the methods that were reported. The main problem in transforming these organisms appears to be in the process of regenerating protoplasts.

Recently (3) we have had considerable success with an unpublished procedure for transformation of B. thuringiensis protoplasts with plasmid DNA (Dietmar Schall, personal communication). We were successful in adapting the procedure to B. cereus 569 and were able to transform protoplasts of that organism with pBC16 and pTV1 DNA. Although we were not successful in reproducibly transforming B. anthracis by this procedure, once we had transformed B. cereus with a plasmid such as pTV1 we could then transfer it to B. anthracis by CP-51-mediated transduction.

Very recently Stepanov, et al. (13) reported their method of cryotransformation of B. anthracis. We have been able to use this method successfully in transforming B. anthracis with pBC16 and pTV1. The procedure

is outlined in the last Annual Report (5). In addition several procedures for electroporation of bacteria have been reported recently and some of these have been applied successfully to B. anthracis and B. cereus (3, 4, 5). Thus the introduction of small plasmids into B. anthracis is no longer a problem. However, in the electroporation and transformation methods plasmid size is a limiting factor; transduction and conjugation remain the most reliable methods for transferring large plasmids among B. anthracis strains.

Transposon mutagenesis of the B. anthracis chromosome

pTV1 is a transposition selection vector developed by Youngman (15). This 12.4-kb plasmid contains the Streptococcus faecalis transposon Tn917, which carries an erythromycin-inducible gene for MLS resistance. The plasmid also carries a chloramphenicol resistance determinant and is temperature sensitive for replication. It has been used to carry out transposon mutagenesis in B. subtilis and other Bacillus species into which it can be introduced by transformation or transduction. The fact that we were able to introduce small plasmids into B. cereus and B. anthracis by transformation or electroporation opened up the possibility of using pTV1 for transposon mutagenesis in B. anthracis.

Introduction of pTV1 into plasmid-free derivatives of B. anthracis provided suitable host strains to determine the ability of Tn917 to insert within the chromosome and thus to utilize transposon mutagenesis as a tool that would facilitate genetic studies of B. anthracis. These studies the following aspects (1989):

1. Induction and selection of Tn917 chromosomal insertions in liquid batch culture.

2. Recovery and phenotypic characterization of auxotrophic mutants resulting from Tn917 chromosomal insertions.

3. Confirmation of insertional mutagenesis by transduction with phage CP-51.

Cells in which transposition of Tn917 and loss of pTV1 had occurred were selected by replica plating colonies to L agar containing selective concentrations of erythromycin and lincomycin and to L agar containing 15 µg of chloramphenicol per ml. Greater than 90% of spores prepared from mutagenized

cultures were generally found to be transposants, i.e., Em^r Lm^r and Cm^s. Em^r Lm^r Cm^s colonies were screened for auxotrophic mutants on minimal agar, and mutant phenotypes were determined by supplementing minimal agar with appropriate nutrients. Approximately 1% of the transposants tested were found to be auxotrophic mutants (one was a pigmented mutant).

To determine whether the mutants resulted from Tn*917* insertions or whether they resulted from coincidental spontaneous mutations, transductions with CP-51 were performed as follows:

(i) CP-51 was propagated on each of the mutants and used to transduce Weybridge A UM23 Ura^- or *B. cereus* 569 UM20-1, respectively, with selection for Em^r Lm^r transductants which were then tested for acquisition of the appropriate mutation. In each instance all of the MLS^r transductants tested (from 25 to 312 MLS^r colonies each) displayed the new mutant phenotype that had been acquired by the original transposant.

(ii) CP-51 was propagated on Weybridge A UM23 Ura^- or *B. cereus* 569 UM20-1 and the respective auxotrophic mutants were transduced with selection for the wild-type allele. These were then tested for sensitivity to Em and Lm. In each instance all of the transductants tested (from 25 to 224) were sensitive to the antibiotics. These experiments show that pTV1 can be used successfully as a vector for transposon mutagenesis in *B. anthracis*.

Investigation of the B. anthracis toxin plasmid, pXO1

Several phenotypes have been attributed to the presence of the 184-kb toxin plasmid, pXO1, in *B. anthracis*. Weybridge A strains cured of pXO1 exhibit changed phenotypes with respect to extent and rate of sporulation, sensitivity to bacteriophage, toxin production, growth on minimal medium, and colony morphology (14). One of our reasons for studying the genetics of pXO1 is to identify the regions of the plasmid responsible for conferring these phenotypic characteristics upon the host strain. Another reason for studying pXO1 is to identify other phenotypes which may be associated with the presence of pXO1 in *B. anthracis*. The methods used for insertional mutagenesis of the *B. anthracis* chromosome employing the transposition selection vector pTV1 were used to isolate mutants carrying transposon insertions in pXO1. Such mutants

have been extremely valuable in studying the biology of the B. anthracis plasmids, pXO1 and pXO2 (3, 4, 5).

Classification of pXO1 from different B. anthracis strains. We reported (3) that Weybridge UM44 exhibited different plasmid-derived phenotypic characteristics from those observed in Weybridge A strains. (It should be recalled that the Trp$^-$ auxotroph, Weybridge UM44, was isolated from the "wild-type" Weybridge strain, and Weybridge A was isolated from "wild-type" Weybridge as a mutant that grew much better than the parent strain on a minimal medium, minimal XO). The two strains, Weybridge UM44 and Weybridge A (and auxotrophs derived from the latter) differed in rate and extent of sporulation at 37°C, phage sensitivity, and growth characteristics on minimal medium. However, UM44-derived strains into which pXO1::Tn917 derivatives from UM23 were introduced showed characteristics similar to those typical of UM23. No apparent differences were observed in BamHI restriction patterns of pXO1 from Weybridge UM44 and pXO1 from Weybridge A UM23. However, there were some differences in the PstI and EcoRI restriction patterns of pXO1 from the two strains. Because of differences observed in the toxin plasmid from the two strains, we have suggested that the plasmid of Weybridge UM44 be designated pXO1 and the one from Weybridge A strains be designated pXO1.1. Similarly, the toxin plasmid from other B. anthracis strains will be designated pXO1.2, pXO1.3, etc., as they are characterized.

Transposon mutagenesis of pXO1.1. Our approach with respect to transposon mutagenesis of pXO1.1 using the transposition selection vector pTV1 has been to isolate MLS-resistant, chloramphenical-sensitive colonies altered in particular plasmid-derived phenotypes. The mutagenesis procedure was the same as that used for chromosomal insertions. Colonies from mutagenized cultures were screened for protective antigen production on halo immunoassay plates incubated in 20% CO_2 or in air (bicarbonate was omitted from those plates incubated in air). The same colonies were also screened for phage (CP-51) sensitivity and sporulation characteristics. Transposants which exhibited altered phenotypes were then tested by DNA-DNA hybridization, with Tn917 DNA as the probe, to determine whether the transposon had inserted into the chromosome or pXO1.1.

As described in annual reports (4, 5) we have isolated a large number of mutants which have Tn917 inserted into pXO1.1. In some instances the pXO1.1::Tn917 derivatives were unstable and generated large deletions; thus,

deletion analysis has also been employed to study the genetics of this toxin plasmid. Several transposants and deletants have been isolated which exhibit altered phenotypes with respect to extent and rate of sporulation, sensitivity to bacteriophage, and toxin production. Restriction analysis has revealed regions of the plasmid which may be involved in conferring some of these phenotypic characteristics. The last two annual reports (4, 5) describe some of these mutants in detail.

Several of the transposants harboring pXO1.1::Tn*917* derivatives are particularly interesting. One of them, Weybridge A UM23 tp62, formed a halo on immunoassay plates incubated either in air or CO_2, suggesting that the mutant did not require CO_2 or bicarbonate for protective antigen synthesis. Subsequent tests showed that tp62 produced more protective antigen both in the presence and absence of CO_2 than the parent strain produced in the presence of CO_2. This mutant has been assigned the phenotype PA^{a+} (protective antigen synthesis in air).

Two transposants, tp49 and tp71, exhibited deletions within pXO1.1::Tn*917* of greater than 100 kb. As determined by the halo immunoassay test as well as tests for protective antigen production in broth, tp49 and tp71 are PA^+ and PA^-, respectively. These transposants also showed an increase in the extent of sporulation at 37°C as compared to the parent strain, UM23. These deletion derivatives are missing many of the fragments normally generated from *BamHI* or *PstI* digestion of wild-type pXO1.1. The sizes of the pXO1.1::Tn*917* deletion derivatives from tp49 and tp71 were determined from the summation of the *BamHI* or *PstI* restriction fragments to be approximately 40.0 kb and 47.5 kb, respectively. DNA-DNA hybridizations were used to determine what toxin structural genes were present on these deletion derivatives and to determine the locations of Tn*917*. The results showed that tp49 contains the structural genes for protective antigen and lethal factor but does not contain the structural gene for edema factor; tp71 apparently contains the structural gene for lethal factor, but not the structural genes for protective antigen and edema factor. As stated above the PA^- and PA^+ phenotypes have been confirmed in actual production tests; tests are now in progress to confirm the phenotypes with respect to production of edema factor and lethal factor.

Generation of Tn*917*-LTV3-tagged pXO1.1 derivatives. The transposition selection vector, pLTV3, was constructed by Camilli, et al. (8). The plasmid

contains a Tn917 derivative, Tn917-LTV3, which carries a promoterless lacZ gene as well as the erm gene, a cat gene, and genes for cloning in E. coli. The plasmid itself is 22.1 kb in size and carries the gene for tetracycline resistance. The purpose for introducing pLTV3 into B. anthracis is to generate more transposon-tagged pXO1.1 derivatives by transposon mutagenesis, thus, increasing our library of insertional mutants and hopefully, creating in vivo lacZ gene fusions. The transposon will also be used to replace simple Tn917 insertions in existing pXO1.1::Tn917 derivatives by selecting for chloramphenicol resistance. These "recombinants" can then be screened for transposon replacement and for gene fusions. pLTV3 was isolated from B. subtilis PY1178 and introduced into Weybridge A UM23-1 by electroporation and also by cryotransformation.

Insertions of Tn917-LTV3 into pXO1.1 from pLTV3 were generated in much the same way as Tn917 insertions were generated from pTV1. In 7 out of 8 independent transposon mutagenesis experiments, approximately 100% of the colonies were MLS-resistant and tetracycline-sensitive indicating that Tn917-LTV3 had transposed either to pXO1.1 or to the chromosome and that the cells were cured of pLTV3. From these experiments several transposants have been isolated which exhibit beta-galactosidase activity and are either PA^+ or PA^-.

Investigation of the B. anthracis capsule plasmid, pXO2

The B. anthracis plasmid pXO2 carries information for synthesis of a D-glutamyl polypeptide capsule. Capsule formation is the only function that has thus far been attributed to this plasmid. B. anthracis strains harboring wild-type pXO2 require CO_2 and bicarbonate for capsule synthesis. When such strains are grown in the presence of CO_2 and bicarbonate the colonies appear very mucoid; in the absence of CO_2 and bicarbonate the colonies appear rough.

In previous studies B. anthracis strains harboring pXO2 were divided into 3 groups with respect to capsule phenotype: (1) Strains that produce capsules only when grown on media containing bicarbonate and incubated in a CO_2-rich atmosphere (Cap^{c+} phenotype); (2) Strains that produce capsules when grown in air in the absence of added bicarbonate or CO_2 (Cap^{a+} phenotype); and (3) Strains that are noncapsulated under all growth conditions and yet retain

pXO2 (Cap$^-$ phenotype). We have shown that spontaneous mutations resulting in the second and third phenotypes are associated with pXO2 (1).

Our recent research in this area has focused primarily on isolation, identification, and characterization of mutants resulting from Tn*917* insertions in *B. anthracis* 4229 UM12 Nal^r. A number of mutants having very interesting phenotypes have been isolated (5).

Introduction of pTV1 into *B. anthracis* 4229 harboring the capsule plasmid pXO2. The temperature-sensitive transposition selection vector pTV1 was introduced into *B. anthracis* 4229 UM12 Nal^r by CP-51-mediated transduction. Transductants confirmed to contain both plasmids, i.e., pXO2 and pTV1 were then used to generate Tn*917* insertions in pXO2. Approximately 64% of MLS-resistant chloramphenicol-sensitive colonies carried Tn*917* in pXO2.

Phenotypes of transposants. MLS^r Cm^s colonies, which were presumed to be transposants cured of pTV1, were observed for capsule phenotypes, Cap^{c+}, Cap^{a+}, or Cap$^-$. Transposants exhibiting the following phenotypes have been isolated: (1) Noncapsulated strains (Cap$^-$) which retained pXO2 but produced rough colonies under all growth conditions. (2) Strains that produced capsules only when grown in media containing bicarbonate and incubated in a CO_2-rich atmosphere (Cap^{c+}). These appeared to be similar to wild-type pXO2-containing strains with the exception of a few which did not grow when incubated in air and a few which appeared to be polypeptide overproducers, resembling *Bacillus licheniformis* in this respect. (3) Strains that produced capsules when grown in air in the absence of bicarbonate (Cap^{a+}). Some of these synthesized capsules both in the presence and absence of CO_2 and a few did not grow in the presence of 20% CO_2.

The pXO2::Tn*917* derivatives in some of the transposants that have the Cap^{a+} phenotype have been observed to undergo deletions; deletions appear to occur more frequently in such strains when they are grown in 20% CO_2. We now have a collection of strains carrying deleted versions of pXO2::Tn*917*. Some of these are Cap$^+$ and others are Cap$^-$. One of them, tp24-17, is very small (probably less than 12 kb) but its host cells are still capable of producing capsules. However, they appear to produce less capsular material than strains carrying wild-type pXO2, suggesting that the deleted form of the plasmid retains the structural genes for capsule synthesis but requires an additional determinant to enhance encapsulation.

As mentioned above, some transposants appear to be polypeptide overproducers. The "stringy" properties of their colonies and their propensity to produce confluent growth (mixture of cells and polypeptide) resemble characteristics of B. licheniformis with respect to polypeptide production. Upon continued incubation the growth on agar plates loses its mucoid characteristic and appears rough and "dry". This is characteristic of B. licheniformis. Examples of transposants having this phenotype are 4229 UM12 tp49 and tp50. Hybridization studies with these two transposants have shown that in tp49 Tn917 is inserted into the plasmid and in tp50 Tn917 is inserted into the chromosome. The observation that colonies of these transposants appear rough and "dry" after a relatively short period of time might suggest production of a D-glutamyl polypeptidase. Such an enzyme has been shown to occur in B. licheniformis, but to our knowledge there has been no report of a similar enzyme in B. anthracis. It is interesting that the two transposants which have similar phenotypes have Tn917 in different locations, on pXO2 in tp49 and on the chromosome in tp50. We used CP-51-mediated transduction to determine whether the insertion of Tn917 is responsible for the unusual properties of these two transposants. The results show that in all instances MLS^r transductants exhibited the specific donor phenotype. To confirm the presence and location of Tn917 in the transductants we carried out DNA-DNA hybridization experiments using ^{32}P-labelled pTV1 DNA as the probe. Results showed that when the donor was tp49 carrying pXO2::Tn917, the transposon was located on pXO2 in the transductants. Transductants which received MLS resistance from chromosomally-marked tp50 did not show Tn917 on pXO2.

To gather further evidence for the location of Tn917 in the transductants referred to above, we looked for spontaneous Cap^- mutants that had lost pXO2 and tested them for MLS resistance. If a transductant carried pXO2::Tn917, as would be expected when tp49 was the donor, then spontaneous Cap^- mutants which had lost pXO2 should be sensitive to MLS antibiotics. Four Cap^- $pXO2^-$ mutants isolated from such transductants were all found to be MLS^s. On the other hand, if a transductant carried Tn917 on the chromosome, as would be expected when tp50 was the donor, then spontaneous Cap^- mutants which had lost pXO2 should retain resistance to MLS antibiotics. Six Cap^- $pXO2^-$ mutants isolated from such transductants were all found to be MLS^r.

The above results suggest very strongly that in both tp49 and tp50 the insertion of Tn917 is responsible for the mutant phenotype. We plan to study these transposants further in an effort to understand how they differ genetically and physiologically from the wild-type parent strain.

Phenotype of transposant 4229 UM12 tp47. B. anthracis 4229 UM12 tp47 is a Cap^{c+} (Cap^+ only in CO_2) mutant which does not grow when incubated in air without added CO_2. When cells are grown in medium containing sodium bicarbonate and incubated in 20% CO_2, the colonies are mucoid. When cells are incubated in air without added CO_2, there is a high frequency of revertants (or suppressed mutants) which grow in air. Such cells are probably more likely to be suppressed mutants than true revertants; they retain their resistance to MLS antibiotics and it seems unlikely that precise excision of Tn917 and insertion at another site could occur at such high frequency.

We have shown that both mutant and suppressed cells of tp47 contain pXO2 but because of problems in obtaining good plasmid preparations from this transposant we have not been able to obtain definite proof that Tn917 is inserted into pXO2 rather than into the chromosome. We believe, however, that pXO2 is in the plasmid rather than the chromosome, and hopefully we will be able to confirm this in the near future.

The reason that 4229 UM12 tp47 does not grow in air remains unclear but it is probably not due to a nutritional requirement for CO_2. It seems more likely that the CO_2 requirement results from a defect in some regulatory gene.

Investigation of Bacillus thuringiensis fertility plasmids and conjugative transfer of B. anthracis plasmids, pXO1 and pXO2

Reports from our laboratory have concerned the identification and characterization of six self-transmissible plasmids from five different subspecies of Bacillus thuringiensis (6, 12). One of these plasmids, designated pXO12 (112.5 kb), was isolated from strain 4042A of B. thuringiensis subspecies thuringiensis. Plasmid pXO12 is capable of mediating its own transfer as well as the transfer of a large range of Bacillus plasmids among strains of Bacillus anthracis, Bacillus cereus, and B. thuringiensis. In addition to conjugal transfer functions, pXO12 also encodes production of the insecticidal toxin known as the delta-endotoxin or the parasporal crystal (Cry^+). Because the B. thuringiensis conjugative plasmids studied in our

laboratory have no known selectable markers, it was necessary to assess indirectly the conjugal activities they confer upon their hosts, i.e., by selecting for the transfer of the B. cereus tetracycline resistance plasmid pBC16 and then examining Tc^r transcipients for acquisition of additional plasmids. Once we were able to introduce the transposition selection vector pTV1 into B. anthracis, B. cereus, and B. thuringiensis we were then able to construct pXO12 derivatives that carried Tn917. The isolation of marked derivatives of pXO12 enabled us to determine the transfer frequency of this plasmid directly during mating.

The widespread occurrence of large self-transmissible plasmids among B. thuringiensis strains suggests that conjugation may be an important means of plasmid dissemination in naturally occurring Bacillus populations (7). In the laboratory, this mating system has provided us with an efficient method of shuttling a wide range of plasmids among B. thuringiensis, B. cereus, and B. anthracis.

Physical analysis of the transferred plasmids (3, 10) suggested that pBC16 was transferred by the process of donation while the large B. anthracis plasmids were transferred by the process of conduction. The transfer of pXO1 and pXO2 involved transposition of the B. thuringiensis transposon Tn4430 from pXO12 onto these plasmids.

Plasmids pXO1 and pXO2 contain Tn4430 after pXO12-mediated mobilization. Plasmid analysis was performed on representative Tox^+ or Cap^+ transcipients to confirm the acquisition of pXO1 or pXO2 in addition to pBC16. These analyses showed that there were a variety of plasmid profiles represented among the transcipients. The majority of Tox^+ or Cap^+ transcipients examined harbored pXO12 (Cry^+) and either pXO1 or pXO2 which on agarose gels appeared to be indistinguishable from the respective plasmids present in the donor strains. However, other transcipients which were Cry^+ and either Tox^+ or Cap^+ contained a single large plasmid migrating above the chromosomal DNA in agarose gels. This suggested the formation of cointegrate plasmids. In contrast to the variability of Tox^+ and Cap^+ transcipients, all Tc^r transcipients inherited plasmid DNA which comigrated with pBC16 from the donor strains.

The high frequency of pBC16 transfer suggests that transfer of this plasmid occurs by donation. In contrast, the lower frequency of transcipients which acquired pXO1 or pXO2 suggests that transfer of these large plasmids may be by conduction; a process which requires physical contact between the

conjugative and nonconjugative plasmid. Since in other conjugation systems physical contact between the fertility plasmid and other mobilizable plasmids is often mediated by transposable elements, we examined pXO1 and pXO2 plasmids before and after mobilization for hybridization to Tn4430. Results showed that Tn4430 hybridized to pXO1 from 6 out of 7 Tox^+ transcipients tested and to pXO2 from 7 out of 8 Cap^+ transcipients tested. There was no detectable hybridization with pXO1 or pXO2 before mobilization. As expected, Tn4430 also hybridized to the presumed cointegrate plasmids.

Plasmid pBC16 is unaltered after transfer. As mentioned earlier the high frequency of transfer of pBC16 suggests that this plasmid is transferred by donation. Since transfer of nonconjugative plasmids by donation does not require physical contact with a fertility plasmid, there should not be any alteration in pBC16 after it has been transferred. To confirm this we examined pBC16 before and after mobilization by pXO12. Restriction analysis of the various pBC16 plasmids confirmed that there were no alterations after transfer, and tests for hybridization with ^{32}P-labelled pXO12 showed that there was no homology between the two plasmids

Generation of cointegrate plasmids of pXO12 and the B. anthracis resident plasmids. From mating mixtures that were screened for cotransfer of the Tox^+ and Cry^+ phenotypes from donors carrying pXO1 and pXO12, some of the transcipients that inherited both phenotypes did not contain pXO1 or pXO12. Instead they contained a plasmid which was higher in molecular weight than either of the two expected plasmids. This suggested that in the course of mating cointegrate plasmids were formed between pXO1 and pXO12. To test the ability of these strains to transfer the large plasmid and pBC16, matings were performed using two independent B. anthracis transcipients as donors to cured strains of B. anthracis. The donor strains harboring the putative pXO1::pXO12 cointegrates transferred pBC16 at frequencies comparable to those of pXO12-mediated transfer of pBC16. However, the proportion of Tc^r transcipients which were also Tox^+, i.e., approximately 50%, was much larger than had been observed previously. In addition, all of the Tox^+ transcipients were also Cry^+. Plasmid analysis revealed that the Tox^+ Cry^+ transcipients inherited a large plasmid that comigrated in electrophoretic gels with the suspected cointegrate present in the donors. None of several Tox^- Cry^- transcipients examined carried a similar large plasmid.

Results analogous to those with pXO1 were also obtained with pXO2. A Cap^+ Cry^+ transcipient which harbored pBC16 and an apparent cointegrate of pXO2 and pXO12 was used as the donor in matings with a derivative Weybridge A UM23 containing no plasmids. pBC16 was transferred at a frequency comparable to that of donor strains containing wild-type pXO12. The proportion of Tc^r transcipients which simultaneously acquired the ability to make capsules was much larger than had been observed previously with donor strains that contained wild-type pXO2 and pXO12. Plasmid analysis of Tc^r Cap^+ Cry^+ transcipients revealed that these strains had inherited, in addition to pBC16, a single large plasmid which migrated at the same rate as the presumed cointegrate plasmid in the donor strain. The large plasmid was not found in several Tc^r Cap^- Cry^- transcipients examined.

These results suggest that pXO12-mediated transfer of the high molecular-weight B. anthracis plasmids pXO1 and pXO2 occurs by conduction. They suggest also that the function of Tn4430 in the mobilization of pXO1 and pXO2 is to mediate the formation of cointegrate molecules between the fertility plasmid pXO12 and the nonconjugative B. anthracis plasmids. The cointegrate plasmid is then transferred to recipient cells where it usually resolves into pXO12 and the respective B. anthracis plasmid. However, our results have shown that in some instances these cointegrate plasmids are stably maintained in transcipient cells.

Investigation of the Bacillus subtilis (natto) fertility plasmid, pLS20

A minor part of our effort during the past five years was spent on characterization of the conjugative plasmid, pLS20, which encodes genes for plasmid transfer among strains of B. subtilis, B. cereus, B. anthracis, and B. thuringiensis (1, 2, 3, 4, 11). Evidence indicating that pLS20 was responsible for plasmid transfer includes: (1) $pLS20^+$ strains, but not $pLS20^-$ strains, functioned as donors of pBC16; (2) Incubation of donor and recipient cultures in the presence of DNase, followed by mating in the presence of DNase did not affect plasmid transfer; and (3) Cell-free filtrates of donor cultures did not convert recipient cells to Tc^r.

Plasmid pLS20 does not carry a known selectable marker. Therefore, it was not possible to select for pLS20$^+$ transcipients. Cotransfer of pLS20 with pBC16 from donors carrying only pLS20 and pBC16 demonstrated that the plasmid is self-transmissible. Acquisition of pLS20 by B. subtilis transcipients rendered this species transfer-proficient, thus providing further proof that pLS20 is conjugative.

To facilitate direct selection for pLS20 transfer, pLS20 derivatives which carry the erythromycin resistance transposon Tn917 as a selectable marker were generated.

Plasmids other than pBC16 were tested for pLS20-mediated transfer. Results showed that pLS20 mediates transfer of the Staphylococcus aureus kanamycin resistance plasmid pUB110 at frequencies comparable to those of pBC16 transfer. Plasmids pBC16 and pUB110 are homologous except for the region occupied by their resistance determinants. Therefore, pLS20-mediated transfer of pUB110 was not unexpected. We also observed cotransfer of the B. subtilis (natto) plasmid pLS19 with pBC16 in intraspecific and interspecific matings. However, the S. aureus plasmids pC194 and pE194 were not mobilized by pLS20.

We have identified a 10.8-kb BglII fragment of the plasmid that is responsible for the transfer proficiency of the host organism. Transposon insertions within this region generated mutant plasmids which showed alterations in the transfer phenotype, and deletions of this region rendered the host organism completely transfer-defective. Cloning of the 10.8-kb fragment into the gram-positive cloning vehicle, pBD64, rendered the host organism transfer proficient, thus confirming that the fragment is necessary and sufficient for conferring conjugal transfer ability on host cells.

Suppression of motility of B. subtilis cells harboring pLS20 is a second plasmid-encoded phenotype that can be attributed to the plasmid. This suppression of motility was observed with cells harboring pLS20 and transposon-tagged derivatives thereof, and reversion to wild-type motility was found in cells harboring deletion derivatives of the plasmids. B. anthracis cells harboring pLS20 appeared much more dense in electron microscopic analysis than cells of the same strain not carrying pLS20, suggesting the presence of some sort of coating on the cells. Clewell et al. (9) reported that E. faecalis cells harboring the pheromone-responsive conjugative plasmid pPD1 produce a "fuzzy" coating thought to be an aggregation substance.

Although pLS20 does not appear to encourage the formation of donor and recipient aggregates in the manner that pPD1 does, perhaps the coating on B. anthracis cells carrying pXO2 is related in some way to the mating event.

A physical map of pLS20 was constructed based on restriction analysis of pLS20, pLS20::Tn917 derivatives, and transfer-defective deletion derivatives. The map is included in the last Annual Report (5).

Investigation of phage TP-21 whose prophage is a plasmid

We have shown that the 46-kb plasmid of Bacillus thuringiensis subsp. kurstaki strain HD-1 is the prophage of a phage which we have named TP-21. This appears to be the first phage with a plasmid prophage described for the genus Bacillus. We have shown that TP-21 is a generalized transducing phage. It is likely that some of the transfer of genetic material attributed by other workers to conjugation-like processes in this strain is the result of TP-21-mediated transduction. TP-21 plaque-forming particles contain greater than 48 kilobase pairs of DNA which appears to be circularly permuted and terminally redundant. We isolated TP-21 lysogens which have the 5.2-kb MLS resistance transposon Tn917 inserted in the prophage. Although insertion of Tn917 rendered some isolates defective, several isolates carrying this element produced viable phage which confer erythromycin resistance upon lysogenized hosts. Results of tests with TP-21::Tn917 demonstrate a broad host range among B. anthracis, B. cereus and B. thuringiensis strains.

TP-21 lysogens were very stable during growth at high temperatures. A mutant of TP-21, TP-21c7, was isolated following nitrosoguanidine mutagenesis of a B. anthracis lysogen; this derivative appears to be temperature sensitive for replication. B. cereus lysogens of TP-21c7 grown at 30°C stably maintain the plasmid prophage. At 42°C strains cured of TP-21c7 could be isolated from broth cultures grown from lysogens. A derivative of TP-21c7 was isolated which has Tn917 inserted. Lysogens of the transposon-tagged derivative, like those of the parental mutant phage, were stable at 30°C but could be cured of the prophage at 42°C.

For many reasons TP-21c7::Tn917 seems as if it should serve as an ideal transposition selection vector. However, several experiments in which it was tested as a mutagenic vehicle were unsuccessful. The reasons for this are not

clear. It is conceivable that Tn917 in the temperature-sensitive mutant is defective in transposition. It is also possible that the mutant is not sufficiently temperature sensitive to be useful as a selection vector. Further experiments will be carried out with TP-21c7::Tn917 and with another temperature-sensitive tagged mutant of TP-21 that has been isolated recently.

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II. List of Publications

Papers

1. **Green, B. D., L. Battisti, and C. B. Thorne.** 1989. Involvement of Tn4430 in transfer of Bacillus anthracis plasmids mediated by Bacillus thuringiensis plasmid pXO12. J. Bacteriol. 171:104-113.

2. **Heemskerk, D. D., and C. B. Thorne.** 1990. Genetic exchange and transposon mutagenesis in Bacillus anthracis. Salisbury Medical Bulletin, Number 68, Special Supplement 63-67.

3. **Koehler, T. M., and C. B. Thorne.** 1987. Bacillus subtilis (natto) plasmid pLS20 mediates interspecies plasmid transfer. J. Bacteriol. 169:5271-5278.

4. **Reddy, A., L. Battisti, and C. B. Thorne.** 1987. Identification of self-transmissible plasmids in four Bacillus thuringiensis subspecies. J. Bacteriol. 169:5263-5270.

Ph. D. Dissertations

1. **Battisti, Laurene A.** Characterization of two self-transmissible plasmids from a strain of Bacillus thuringiensis subsp. thuringiensis. Ph. D. Dissertation. University of Massachusetts, Amherst. February 1987.

2. **Green, Brian D.** Mobilization of the Bacillus anthracis plasmids pXO1 and pXO2 by the Bacillus thuringiensis fertility plasmid pXO12. Ph. D. Dissertation. University of Massachusetts, Amherst. February 1988.

3. **Jaworski, Deborah D.** Physical and genetic analysis of the Bacillus subtilis (natto) fertility plasmid pLS20. Ph. D. Dissertation. University of Massachusetts, Amherst. May 1990.

4. **Koehler, Theresa M.** Plasmid-related differences in capsule production by Bacillus anthracis and Characterization of a fertility plasmid from Bacillus subtilis (natto). Ph. D. Dissertation. University of Massachusetts, Amherst. September 1987.
5. **Reddy, Amula.** Characterization of four self-transmissible Bacillus thuringiensis plasmids. Ph. D. Dissertation. University of Massachusetts, Amherst. February 1987.
6. **Ruhfel, Robert E.** Physical and genetic characterization of the Bacillus thuringiensis subsp. kurstaki HD-1 extrachromosomal temperate phage TP-21. Ph. D. Dissertation. University of Massachusetts, Amherst. September 1989.

Abstracts

1. **Heemskerk, D. D., and C. B. Thorne.** 1988. Physical and genetic analysis of the Bacillus subtilis (natto) fertility plasmid pLS20. Abstr. Annu. Meet. Am. Soc. Microbiol. H-3.
2. **Heemskerk, D. D., and C. B. Thorne.** 1989. Genetic analysis of the Bacillus subtilis (natto) fertility plasmid pLS20. Abstr. Annu. Meet. Am. Soc. Microbiol. H-269.
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8. **Ruhfel, R. E., and C. B. Thorne.** 1988. Physical and genetic characterization of the Bacillus thuringiensis subsp. kurstaki HD-1 extrachromosomal temperate phage TP-21. Abstr. Annu. Meet. Am. Soc. Microbiol. H-4.

9. **Ruhfel, R. E., and C. B. Thorne.** 1989. Isolation of a temperature-sensitive replication mutant of phage TP-21 and a Tn917-tagged derivative. Abstr. Annu. Meet. Am. Soc. Microbiol. H-211.

III. List of Personnel

Principal Investigator: C. B. Thorne, Professor

The following graduate students in pursuit of M. S. and Ph. D. degrees worked on the project:

Battisti, Laurene
Green, Brian D.
Guaracao-Ayala, Ana
Heemskerk (Jaworski), Deborah
Hornung, Jan
Koehler, Theresa M.
Reddy, Amala
Ruhfel, Robert E.

The following were employed as technicians for part of the contract period:

Coyne, Michael
Hagan, Deborah
Hornung, Jan

The following undergraduate students were employed on an hourly basis for various periods of time as laboratory workers:

Beck, Robert	Lazazzera, Beth
Cartagena, Maria	McVeigh, John
Chen, Laura	Ortiz, Sarah
Coyne, Michael	Ramin, Rahimi
Freedmen, Matthew	Wilcox, Heidi
Garcia, Virna	Zedicker, Walter

IV. List of Those Who Received Graduate Degrees While Working on the Contract:

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Reddy, Amala, Ph.D.

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